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The Journal OF Biological Chemistry

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AUTHOR INDEX

The Journal of Biological Chemistry, founded in 1905, is designed for the prompt publication of original investigations of a chemical nature in the biological sciences.

All manuscripts should be addressed to Dr. John T. Edsall, *Editor, The Journal of Biological Chemistry*, 10 Divinity Avenue, Harvard University, Cambridge 38, Massachusetts.

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Instructions To Authors

Submission of a manuscript to the Editor involves the tacit assurance that no similar paper, other than an abstract or preliminary report, has been, or will be submitted for publication.

Form and Style of Manuscript

Carelessness in the preparation of a manuscript only leads to delay in publication and to waste of time on the part of Editors and Referees. An improperly prepared manuscript must be returned to the author for correction of technical faults regardless of its scientific merit. Accordingly, it is important that all contributions should be carefully examined before being submitted, to make sure that they conform as closely as possible to the following instructions.

Manuscripts should be typed with double or triple spacing throughout (including references), and the original copy should be submitted along with one clear carbon copy. Before the manuscript is mailed to the Editor all errors in typing should be corrected, and the spelling of proper names and of words in foreign languages, the accuracy of direct quotations and bibliographical references, and the correctness of analytical data, as well as of numerical values in tables and in the text, should be carefully verified by the author. Care in grammatical construction is essential; vague, obscure, and ambiguous statements must be avoided. Since the *Journal* is read by scientists in foreign countries, technical neologisms and "laboratory slang" should not be used; when unavoidable, such terms should be defined. Variations from standard nomenclature and all arbitrary abbreviations should be explained. The forms of spelling and abbreviation used in current issues of the *Journal* should be employed, and for chemical terms the usage of the American Chemical Society as illustrated by the indexes of *Chemical Abstracts* should be followed. A number of capital letter abbreviations for substances which are frequently referred to in biochemical publications are widely used and generally understood.

A list of approved abbreviations, which may be used without further definition in articles in the *Journal*, will be found in the article on "Use of Abbreviations" on pages 3 and 4. Other abbreviations should be used sparingly, and must be defined by each author in a footnote at the point where the first such abbreviation occurs. Ordinarily no abbreviations should be used in summaries. For further details, see "Use of Abbreviations."

Separate sheets should be used for the following: (*a*) title, (*b*) author(s) and complete name of institution or laboratory, (*c*) running title, (*d*) references, (*e*) footnotes, (*f*) legends for figures, (*g*) tables, and (*h*) other subsidiary matter. When an elaborate mathematical or chemical formula (one which cannot be printed in single horizontal lines of type) appears in the text, a duplicate of it should be typed on a separate sheet. All such supplementary sheets, except the title, author(s), and running title pages, should follow the text, and all sheets should be numbered in succession, the title page being page one. Manuscripts that do not conform to these instructions will have to be cut and rearranged by the printer so that the matter to be set in different type sizes can be separated.

Title

The title should be as short as is consistent with clarity; in most instances two printed lines are adequate to give a clear indication of the subject matter of the paper. The title should not include chemical formulas or arbitrary abbreviations, but chemical symbols may be used to indicate the structure of isotopically labeled compounds. A running title should be provided (not to exceed 60 characters and spaces).

Organization of Manuscript

A desirable plan for the organization of a paper is the following: (*a*) introductory statement, with no heading, (*b*) "*Experimental Procedure*" (or "*Methods*"), (*c*) "*Results*," (*d*) "*Discussion*," (*e*) "*Summary*," (*f*) "*References*." The approximate location of the tables and figures in the text should be indicated in the margin. Any general acknowledgments that are to be made should be placed after the *Summary*, just preceding the *References*. Mention of more specific instances of acknowledgment may be made in footnotes.

1. The introductory paragraphs should state the purpose of the investigation and its relation to other work in the same field, but extensive reviews of the literature should not be given. A brief statement of the principal findings is helpful to the reader.

2. The description of the experimental procedures should be as brief as is compatible with the possibility of repetition of the work. Published procedures, unless extensively modified, should be referred to only by citation in the list of references.

3. The results are customarily presented in tables or charts and should be described with a minimum of discussion.

4. The discussion should be restricted to the significance of the data obtained. Unsupported hypotheses should be avoided.

5. *Every paper must conclude with a brief summary* in which the essential results of the investigation are succinctly outlined.

6. The references should conform to the style used in current issues of the *Journal*. In the case of books, the authors' names with initials, the title in full, the edition if other than the first, the publisher, the place of publication, the year of publication, and the page should be cited, in this order. Responsibility for the accuracy of bibliographic references rests entirely with the author; all references should be confirmed by comparison of the *final manuscript* with the original publications. Mention of "unpublished experiments," "personal communications," etc., must be made in footnotes, and not included in the *References*. References to papers which have been *accepted* for publication, but which have not appeared, should be cited just as other references, with the abbreviated name of the journal followed by the words "in press." It is advisable that copies of such papers be submitted to the Editors whenever the findings described in them have a direct bearing on the paper for publication.

7. If the paper submitted is one of a series, the immediately preceding paper of the series should be included in the references, and identified as such, either in the text or in a footnote near the beginning of the paper.

Chemical and Mathematical Formulas

Reference in the text to *simple* chemical compounds may be made by the use of formulas when these can be printed in single horizontal lines of type. The use of structural formulas in running text should be avoided. Chemical equations, structural formulas, and mathematical formulas should be centered between successive lines of text. Unusually complicated structural formulas or mathematical equations which cannot conveniently be set in type should be drawn in India ink on a separate sheet in form suitable for reproduction by photoengraving (examples, *J. Biol. Chem.*, **228**, 612, 630, 714, 753 (1957)).

Tables

For aid in designing tables in an acceptable style, reference should be made to current issues of the *Journal*. A table should be constructed so as to be intelligible without reference to the text. Only essential data should be tabulated. Every table

should be provided with an explanatory caption, and each column should carry an appropriate heading. Units of measure must always be clearly indicated. If an experimental condition, such as the number of animals, dosage, concentration of a compound, etc., is the same for all of the tabulated experiments, this information should be given in a statement accompanying the table, and not in a column of identical figures in the table.

The presentation of large masses of essentially similar data should be avoided, and, whenever space can be saved thereby, statistical methods should be employed by tabulation of the number of individual results and the mean values with their standard deviations or the ranges within which they fall. A statement that a significant difference exists between the mean values of two groups of data should be accompanied by the probability derived from the test of significance applied.

Only in exceptional cases, the necessity for which must be clearly demonstrated, may the same data be published in two forms, such as in a table and a line figure.

Illustrations

The preparation of illustrations is particularly important, and authors are requested to follow carefully the directions given below. In case of doubt, the Editorial Office will gladly supply specific information.

It is helpful to the Editorial Office if all charts and drawings are submitted on sheets 8½ × 11 inches in size. Large sized drawings or those much smaller than manuscript sheets are difficult to handle, and the Editor reserves the right to return unsuitable drawings to the author with a request for new drawings which conform with the requirements for publication.

Drawings that have been prepared for presentation as lantern slides are frequently unsuitable, since the artist is often instructed to include information which should properly appear in the legend of the published figure.

Charts should be planned so as to eliminate all waste space and, when several figures are submitted, should be designed so that two figures can be printed side by side where appropriate. In general, only one figure should be drawn on a sheet, and ample margin should be provided for labeling and for instructions about reproduction added in the Editorial Office. All drawings should be prepared in the same style with respect to lettering, weight of lines, indications of points of observation, etc.

The scales used in plotting the data should be so chosen as to avoid waste of space, especially vertical space. Tall, narrow drawings should be avoided, as should also low wide drawings. Curves that can be placed on one chart without undue crowding should not be given in separate charts. The drawings should be made on Bristol board, blue tracing cloth, or on coordinate paper *printed in light blue*. Mounting on heavy cardboard is undesirable. Photoengravings made from photographic prints are inferior to those prepared from the original drawings, which should, therefore, be submitted whenever possible. If it is necessary to submit photographic prints because of the excessive size of the originals, these should be carefully prepared. All parts of the chart should be in even focus, and rules and lettering should be fairly thick, as well as large enough for the necessary reduction. *When oversize original drawings are submitted, a set of small photographic prints must also be included for the use of Referees.* A duplicate set of figures must accompany the carbon copy of the manuscript. These need not be of the same quality as the original figures intended for publication, but must be clear and legible for the use of Referees.

All charts should be ruled off on all four sides close to the area occupied by the curves, and descriptive matter placed on the ordinate and abscissa should not extend beyond the limits of these rules. Black India ink should be used throughout. Letters and figures should be uniform in size and style and large enough so that no character will be less than 1.5 mm. high after reduction. As a rule, the printed figure is one-half or one-third the size of the original drawing, but oversize drawings must be reduced still further. Drawings which contain letters or characters which do not permit of such reduction must be returned to the authors with a request that the size of the lettering be increased.

The scales used in plotting the data should be indicated by short index lines perpendicular to the marginal rules of the drawings on all four sides, unless more than one scale is used on the ordinates, at such intervals that interpolation will permit reasonably accurate evaluation of experimental points. Points of observation should be indicated by symbols drawn with instruments. The significance of the symbols should be explained on the chart or in the legend. If they are not explained on the face of the chart, only standard characters, of which the printer has type, should be employed (×, ○, ●, □, ■, △, ▲, ◓).

Photographs submitted for half-tone reproduction should be printed on white, glossy paper. The cost of half-tone reproductions will be charged to the authors.

Each chart, graph, and illustration should be clearly identified with a soft pencil on the margin, with the authors' names, the number of the figure, and, if necessary, an indication of "top." Each figure must have an explanatory legend. Legends should not be attached to or written on the illustration copy.

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Authors are responsible for the reading of galley proof. Printers' errors should be marked in red on the proofs. Changes made by the authors should be marked in black. The cost of changes, other than correction of printers' errors, will be charged to authors. Changes of text made in galley proof, other than printers' errors, are very expensive and should be made only when vitally necessary in the interests of accuracy or clarity. Arbitrary or trivial changes will not be accepted by the Editor. Newly obtained data cannot be inserted. However, where the desirability or necessity of the addition of a "note added in proof" can be demonstrated to the satisfaction of the Editor, the manuscript of such a note may be attached to the proof. This addition must then receive the approval of the Editor.

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Use of Abbreviations

The *Journal* recognizes the utility and convenience of some nonstandard abbreviations for chemical substances, particularly in equations, tables, or figures requiring several unwieldy terms in close or repeated context. The *Journal* therefore will accept a limited use of abbreviations of specific meaning as defined below. However, in order that scientific writing be comprehensible to the reader, clarity and unambiguity of expression must remain prime considerations.

Titles and summaries, which are most often translated into foreign languages, should be free of abbreviations, and abbreviations should be minimized in the introductory sections. In the remainder of the manuscript, abbreviations may be used in the text sparingly and only if advantage to the reader results. Chemical equations, which traditionally depend upon abbreviations, may utilize an abbreviation for a term which appears in full in the neighboring text. This latter procedure is also permissible in summaries, when necessary to avoid excessively cumbrous expressions, as in equations or polymer structures. Such essential abbreviations in summaries would be defined therein when first used; *e.g.* flavin adenine dinucleotide (FAD).

Formulation of Abbreviations—Abbreviations other than those listed or defined below should be in accordance with the following principles:

The number of these introduced per article should be limited (3 or 4); none should be introduced except where repeated use is required (see above). Three letters are considered optimum in length. The formation of words by these letters or duplication of an accepted abbreviation is to be avoided. Where a number of derivatives, salts, or addition compounds may be formed, the root or common basic structure should be the one abbreviated so that appendages may be hyphenated to it (*e.g.* AMP-sulfate, acyl-AMP). It is suggested that abbreviated names be hyphenated throughout, even where the full name is not (*e.g.* glucose-6-P, but glucose 6-phosphate). Structural analogues of a given compound are not to be abbreviated as if they were derivatives of that compound (*e.g.* the nicotinic acid analogue of DPN). Accepted symbols for elements or radicals are recommended where applicable (*e.g.* glycero-P for glycerophosphate, P-glycerate for phosphoglycerate).

Names Ordinarily Not To Be Abbreviated—Names of enzymes (except when named in terms of an abbreviation, *e.g.* glucose-6-P dehydrogenase, ATPase, but glucose 6-phosphatase; RNase, DNase), pyridoxal, pyridoxamine, deoxypyridoxine, thiamine, cocarboxylase, pantothenate, folic acid, pteroylglutamate, trichloroacetic acid, perchloric acid, the tricarboxylic acid cycle and members thereof. Abbreviations for pteroyl-L-glutamic acid derivatives (but not the substance itself) may use PGA for the parent compound (*e.g.* PGA-H_4; N^5-formyl-PGA-H_4; hydroxymethyl (or CH_2OH-) PGA-H_4). (Note that pteroyl-L-glutamic acid is one member of the class of compounds known collectively as the folic acids.) However, since PGA has also been used by some authors to denote phosphoglyceric acid or polyglutamic acid, it is essential that this abbreviation be defined in a footnote in each paper in which it is used.

Accepted Abbreviations—The abbreviations in the list given below, may be used without definition. Other abbreviations should be defined in a single footnote at the point of introduction of the first one. Accepted abbreviations are as follows (*cf. Biochem.* J., **66**, 8 (1957)):

DPN (or DPN^+), DPNH	diphosphopyridine nucleotide and its reduced form
TPN, TPNH	triphosphopyridine nucleotide and its reduced form
FAD, $FADH_2$	flavin adenine dinucleotide and its reduced form
NMN	nicotinamide mononucleotide
GSH, GSSG	glutathione and its oxidized form
CoA, acyl-CoA	coenzyme A and its acyl derivatives (*e.g.* acetyl, etc.)
AMP, GMP, IMP, UMP, CMP	the 5′-phosphates of ribosyl adenine, guanine, hypoxanthine, uracil, cytosine
2′-AMP 3′-AMP (5′-AMP), etc.	the 2′-, 3′-, (and 5′-, where needed for contrast) phosphates of the nucleosides
ADP, etc.	the 5′(pyro)-diphosphates of adenosine, etc.
ATP, etc.	the 5′(pyro)-triphosphates of adenosine, etc.
deoxy-AMP(dAMP, dGMP, dIMP, dUMP, dCMP, dTMP)*	the 5′-phosphates of 2′-deoxyribosyl adenine, etc.

Phosphorylated derivatives may be depicted as phosphate derivatives of the parent compounds with P- or -P representing phosphate, as in glucose-6-P, P-glycerate, glycerol-P, P-enolpyruvate, PP-ribose-P (ribosylpyrophosphate 5-phosphate).

RNA, DNA	ribonucleic acid, deoxyribonucleic acid
RNase, DNase	ribonuclease, deoxyribonuclease
UDP-glucose, UDP-galactose, etc.	uridine diphosphate glucose, galactose, etc.

For Diagrammatic Representation of Polymers or Sequences—(*a*) For the amino acid residues in polypeptides, the residue with the free alpha-amino group (if one is present) to be understood to be at the left of the sequence as written (*Ann. Rev. Biochem.*, **16**, 224 (1947)):

Gly, Ala, Val, Leu, Ileu	glycyl, alanyl, valyl, leucyl, isoleucyl,
Pro, Phe, CySH, CyS-, Met,	prolyl, phenylalanyl, cysteinyl, half-cystyl, methionyl,
Try, Arg, His, Lys, Asp, Glu,	tryptophyl arginyl, histidyl, lysyl, aspartyl, glutamyl,
Glu-NH_2, Asp-NH_2, Ser,	glutaminyl, asparaginyl, seryl,
Thr, Tyr, Hypro, Hylys	threonyl, tyrosyl, hydroxyprolyl, hydroxylysyl

These symbols should be separated from each other by periods (*e.g.* Gly.Val.Asp.Ser). Groups of residues of unknown sequence may be enclosed in parentheses and separated by commas. These abbreviations should be used only for amino acid residues in peptide linkage; never for the free amino acids, the names of which should be written out in full.

* When it is necessary to use d for deoxy, it should be so defined. The full prefix requires no such definition.

(*b*) For the polysaccharides (*J. Chem. Soc.*, **1952**, 5121; *Chem. Eng. News*, **31**, 1776 (1953)):

G†, Fru, Gal, Rib, deoxy-Rib (dRib), etc.*	glucose, fructose, galactose, ribose, deoxyribose, etc.

(These abbreviations are to be used only for derivatives or polymers of the monosaccharides, not for the free monosaccharides themselves, the names of which should be written out in full.) When it is necessary to indicate furanose, pyranose, etc., the letter *f* or *p*, following the saccharide abbreviation, may be used. Thus, Rib*f* for ribofuranose. To indicate a uronic acid, the suffix A, for acid, may be used. Thus, GA for glucuronic acid, GalA for galacturonic acid. To indicate a 2-amino-2-deoxysaccharide, the suffix N is added to the parent saccharide symbol, and an *N*-acetyl derivative is shown by NAc. Thus, GalN for galactosamine, GalNAc for N-acetyl galactosamine. Configuration symbols (L-, D-) may be used as prefixes. Each residue is separated from the next by a hyphen; thus, UDP-GalNAc.

(*c*) For polynucleotides of specific structure, the letter p to the left of the nucleoside initial indicating a 5′-phosphate; the letter p to the right, a 3′-phosphate:‡ Thus, for polyribonucleotides (A, G, etc., representing the nucleosides of adenine, guanine,† etc.):

pApG	5′-*O*-phosphoryl-adenylyl-(3′-5′)-guanosine or guanylyl-(5′-3′)-adenosine 5′-phosphate
ApGp	adenylyl-(3′-5′)-guanosine 3′-phosphate
ApG-cyclic-p	adenylyl-(3′-5′)-guanosine 2′:3′-phosphate

for polydeoxyribonucleotides:*

d-pApGpT	5′-*O*-phosphoryl-deoxyadenylyl-(3′-5′)-deoxyguanylyl-(3′-5′)-deoxythymidine, or deoxythymidylyl-(5′-3′)-deoxyguanylyl-(5′-3′)-deoxy-adenosylyl 5′-phosphate.

† Confusion between G for guanosine and for glucose, if it occurs, should be avoided by the use of one name in full.

‡ For further examples of this system of abbreviation see, for instance, Heppel, Ortiz, and Ochoa, *J. Biol. Chem.*, **239**, 679, 695 (1957), and especially Gilham and Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).

UNITS OF MEASUREMENT

Units of Mass

kilogram	kg.
gram	gm.
milligram	mg.
microgram	μg. (*not* γ)
millimole	mmole (*not* mM)
micromole	μmole (*not* μM)

Units of Concentration

molar (mole/liter)	M
millimolar	mM
micromolar	μM

Units of Length, Area, Volume, etc.

meter	m.
centimeter	cm.
millimicron	mμ
Angstrom (10^{-8}cm.)	A
square centimeter	$cm.^2$
cubic centimeter	cc., or $cm.^3$
liter	l.
milliliter	ml.
microliter	μl. (*not* λ)
sedimentation coefficient	*s.*
sedimentation coefficient in water at 20°, extrapolated to zero concentration	$s^0_{20,w}$
Svedberg unit of sedimentation coefficient (10^{-13} sec.)	S
diffusion coefficient (usually given in $cm.^2$/sec.)	*D*

Terms Used in Reporting Spectrophotometric Data—Because there are several ways of reporting spectrophotometric data and some conventions are not generally understood, it is essential for an author to indicate the relation between the symbols used. It is recommended that Beer's law be stated with one of the following sets of symbols.

$$A \equiv OD = -\log_{10} T = a_M Cb \equiv \epsilon Cb = a_s cb$$

In these equations A is absorbancy (preferred), OD is optical density, **T** is transmittancy (not transmittance, as for a plate of glass), C is the concentration of the absorbing substance in moles per liter, c is its concentration in other units (which *must be specified*), a_s is the absorbancy index, a_M is the molar absorbancy index (identical with ϵ, the molar extinction coefficient), and b is length of the optical path in cm. If Beer's law is not applicable to a particular substance in solution, this should be explicitly stated; even in such cases the substance may be characterized by reporting the absorbancy at a specified concentration.

When a substance is characterized by a molar absorbancy index by use of radiant energy which is not confined strictly (as in a line spectrum) to the wave-length or frequency specified, the exact value of this index will be somewhat ambiguous unless the so-called spectral interval isolated is also reported.

See, for instance, K. S. Gibson, *Spectrophotometry (200 to 1,000 millimicrons)* U. S. Department of Commerce, National Bureau of Standards, Circular 484, issued September 15 (1949), U. S. Gov't Printing Office, Washington, D. C.; also, W. M. Clark, *Topics in Physical Chemistry*, Second Edition, pp. 646–657, The Williams & Wilkins Co., Baltimore, 1952.

Equilibrium and Velocity Constants

Dissociation constants, association constants, and Michaelis constants should ordinarily be written in terms of concentrations in moles per liter; for instance, for the reaction $Mg^{++} + ATP^{4-} \rightleftharpoons MgATP^{2-}$, the association constant is: $K = (MgATP^{2-})/(Mg^{++})(ATP^{4-})$; (in units of M^{-1}).

If other units of concentration are employed, they should be clearly indicated at the point where the equilibrium constant is defined, and where its value is given.

Values of velocity constants should be similarly specified, first order velocity constants being generally given in $sec.^{-1}$ (other units of time may be used on occasion, but in any case the time unit should be specified). Second order velocity constants are ordinarily given in M^{-1} $sec.^{-1}$.

The term milligram per cent (mg.%) should not be used. Weight concentrations should be given as gm. per ml., gm. per 100 ml., gm. per l., etc.

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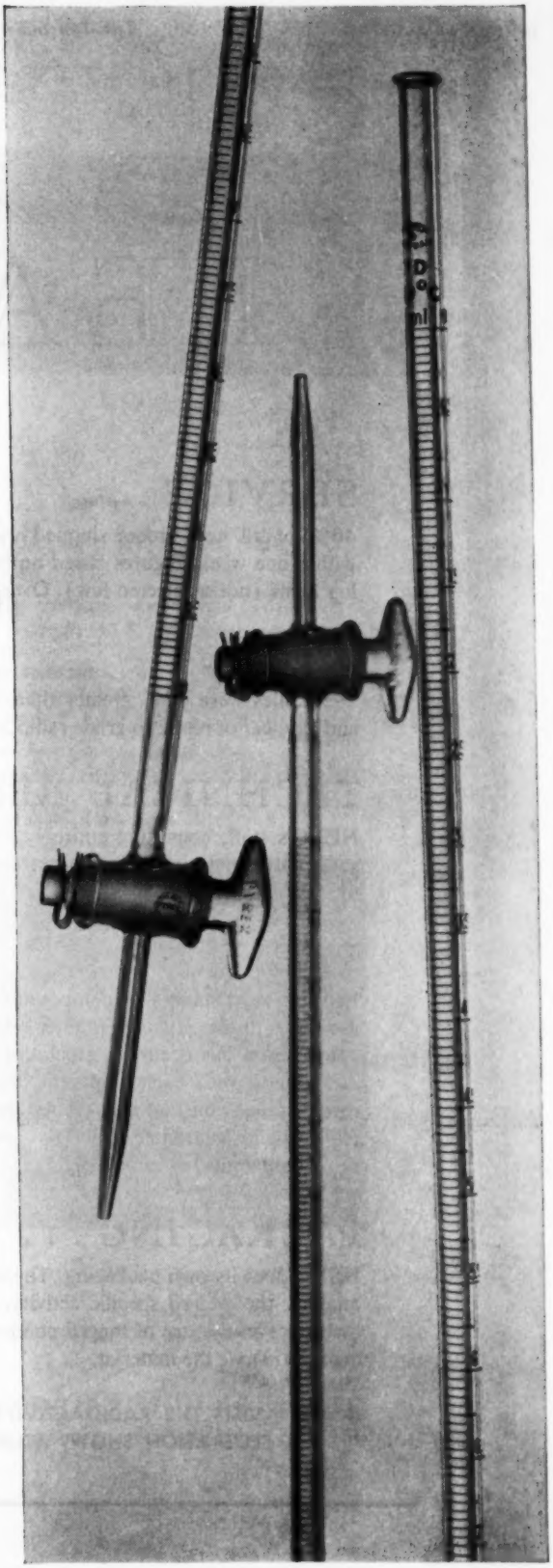

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For fast reproducible determinations of melting points conforming to official U.S.P. and similar methods using glass capillary tubes. Electrically heated, range from room temperature to 350°C. Takes up to five samples simultaneously.

Consisting of illuminated, silicone fluid bath with electric stirrer, lagless heater controlled by autotransformer, built-in capillary vibrator, armored thermometer, etc., combined in a single sturdy unit.

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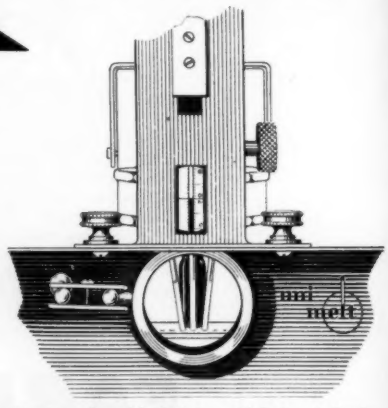
Upper mirror assembly of periscope is raised or lowered to follow mercury level of thermometer by turning knob at side of reader housing.



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Safety—Bath enclosed in cabinet except for observation port which is shielded by the magnifier. Removal of bath automatically disconnects heater and stirrer.

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6406-H. Showing bath unit lifted out of cabinet

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The Formation of 2-Keto-3-deoxyheptonic Acid in Extracts of *Escherichia coli* B

I. IDENTIFICATION

ARTHUR WEISSBACH AND JERARD HURWITZ

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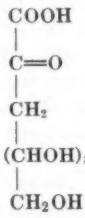
(Received for publication, April 1, 1958)

Waravdekar and Saslaw (1) have reported a sensitive color test for deoxyribose which depends on the periodate oxidation of the deoxy sugar. The malondialdehyde formed in this oxidation is coupled with thiobarbituric acid to give a chromogen with an absorption maximum at 532 mμ. While investigating the metabolism of ribose 5-phosphate in extracts of *Escherichia coli* strain B, the formation and accumulation of a product which reacted in the periodate-thiobarbituric acid test was noticed. The color formed in this case had an absorption maximum at 545 to 550 mμ rather than 532 mμ. The compound responsible for the color has been identified as 2-keto-3-deoxyheptonic acid (I) in which the configuration of the groups on carbons 4, 5, and 6 remains undetermined.



COMPOUND I

This paper is to report the partial identification and properties of this compound. The following paper will deal with its enzymic synthesis.

EXPERIMENTAL

Enzymic Preparations—*E. coli* strain B was grown in liquid media containing 13.6 gm. of KH_2PO_4, 2.0 gm. of $(NH_4)_2SO_4$, 0.2 gm. of $MgSO_4 \cdot 7\ H_2O$, 0.01 gm. of $CaCl_2$, 0.0005 gm. of $FeSO_4 \cdot 7\ H_2O$ and 5 gm. of glucose per liter. The pH was adjusted to 7.2 with KOH and the culture incubated at 37° with vigorous aeration. When the growth was sufficient to give an optical density of 0.200 to 0.300 at 650 mμ, as measured in the Coleman Junior spectrophotometer, the cells were collected in the Sharples supercentrifuge. After being washed with water, the cells were broken by Nossal disintegration (2) at −10°. The cells were shaken for a total of 1 minute in 15-second periods. For every 2 gm. of cells, 8 gm. of glass powder and 8 ml. of 0.1 M Tris[1] buffer, pH 7.5, were used. After the cells had been broken, the mixture was centrifuged at 20,000 × *g* and the clear supernatant fluid was used as the cell-free extract (crude extract).

Preparation of Dialyzed Extract—The cell-free extract was brought to 90 per cent saturation with ammonium sulfate. The precipitate was centrifuged, collected, and dissolved in one-half the original extract volume using 0.1 M Tris, pH 7.5. This ammonium sulfate fraction was dialyzed for 6 hours against 0.01 M sodium acetate containing 0.0001 M ethylenediaminetetraacetic acid in a rocking dialyzer at 0°.

Determinations

The thiobarbituric acid test (1) was used with the following modifications. The sample in 0.20 ml. or less of solution was added to 0.25 ml. of 0.025 N HIO_4 in 0.125 N H_2SO_4. After 20 minutes at room temperature, 0.50 ml. of 2 per cent sodium arsenite in 0.5 N HCl was added with shaking, and the solution was permitted to stand two minutes. Two ml. of 0.3 per cent thiobarbituric acid (pH 2) were added and, after stirring, the mixture was heated at 100° for 10 minutes. When the mixture had cooled, the optical density was measured at 548 mμ in the Beckman model B spectrophotometer. Protein was determined by the method of Bücher (3). α-Keto acids were measured by the semicarbazide method (4). Formaldehyde was determined with chromotropic acid (5). Formic acid was determined enzymatically by the method of Rabinowitz and Pricer (6)[2]. Lactones were measured by the method of Hestrin (7).

Phosphatase Incubations—The reaction mixtures contained 2 μmoles of phosphate ester, 40 μmoles of acetate buffer, pH 5.1, 1 μmole of $MgCl_2$, 10 units of potato acid phosphatase in a total volume of 0.36 ml. Incubations were carried out at 37° for 120 to 180 minutes.

Chromatography—Descending chromatograms with Whatman No. 1 filter paper were employed. The solvents were acetone-water (85:15), phenol saturated with water, ethyl acetate-pyridine-water (2:1:2), methanol-formic acid-water (8:13:7), propanol-formic acid-water (6:3:1), and concentrated ammonia-methanol-water (1:6:3). Compounds were visualized with alkaline silver nitrate (8) or with a semicarbazide spray (4).

Substrates—Ribose 5-phosphate was obtained from Schwarz Laboratories, Inc., ATP and DPN from the Sigma Chemical Company, mercaptoethanol from Eastman Organic Chemicals,

[1] The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; KDA, 2-keto-3-deoxyheptonic acid; KDPA, 2-keto-3-deoxy-7-phosphoheptonic acid.

[2] We are indebted to Dr. Jesse Rabinowitz and Mr. William E. Pricer, Jr., for the formate determinations.

2-deoxy-D-ribose from Mann Research Chemicals, Inc., 2-deoxy-D-glucose from Nutritional Biochemicals Corporation, 1-C^{14}-ribose was obtained from Dr. H. Isbell of the National Bureau of Standards. 1-C^{14}-ribose 5-phosphate was prepared by the method of Horecker *et al.* (9). Metasaccharinic acid was the kind gift of Dr. J. C. Sowden.

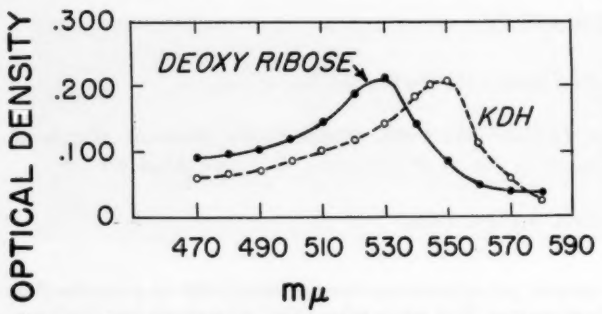


FIG. 1. The absorption spectra of deoxyribose and 2-keto-3-deoxyheptonic acid (KDH) in the thiobarbituric acid test.

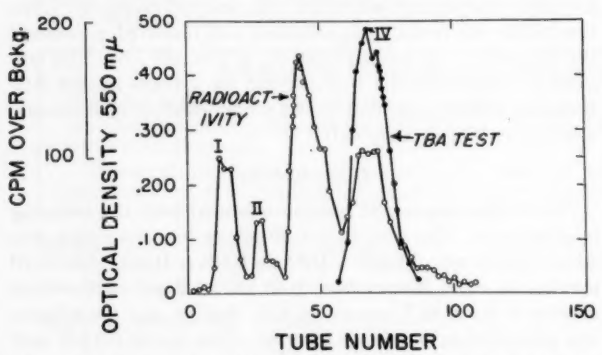


FIG. 2. Assay for C^{14} activity and for color in the thiobarbituric acid (TBA) test.

TABLE I

Periodate degradation of C^{14}-Compound I

	μmole/ml.
α-Keto acid*	8.25
HCHO formed†	8.6
HCOOH formed‡	18.7

* Determined by the semicarbazide method using pyruvic acid as a standard (4).

† 0.10 ml. of C^{14}-Compound I and 1.0 ml. of 0.025 M periodate in 0.125 N H_2SO_4 were incubated at room temperature for 2 hours. After the addition of 0.05 ml. of 1 M $SrCl_2$ (19), the pH was adjusted to 6 with 0.1 ml. of 1.0 N NaOH. After 1 hour at 0° the precipitate was centrifuged, washed with water, and the washings were combined with the original supernatant. Total volume = 5.0 ml. 0.25 ml. of this solution was treated with 0.05 ml. of 2 per cent sodium arsenite and assayed for HCHO.

‡ 0.050 ml. of C^{14}-Compound I was incubated with 0.50 ml. of 0.025 M periodate in 0.125 N H_2SO_4, for 2 hours at room temperature. 1.0 ml. of 2 per cent sodium arsenite in 0.5 N HCl was added and the solution was then steam-distilled until 120 ml. of distillate had been collected. 0.1 N Na_2SO_3 was added until the faint iodine color was absent (0.20 ml.). The pH was adjusted to 7.4 with NaOH and the solution was concentrated in a vacuum to 5.25 ml. Aliquots were assayed for formate.

Results

Incubation of the crude cell-free extract of *E. coli* with ribose 5-phosphate leads to the formation of a compound (I) which produces a color in the thiobarbituric acid test. In Fig. 1 the absorption spectrum of the color produced by this compound is shown with the color produced by deoxyribose. The colored compound formed from Compound I in the thiobarbituric acid test shows other characteristics which indicate that it is not derived from malondialdehyde. Color formation reaches a maximum in about 10 minutes and then decreases after that time. This is in contrast to the color produced by malondialdehyde which is maximal in about 20 minutes at 100° and is stable. Furthermore, the color produced by malondialdehyde is stable in strong alkali whereas the color produced by Compound I is alkali-labile.

Accumulation and Isolation of Compound I

Compound I was prepared from 1-C^{14}-ribose 5-phosphate. Since the formation of Compound I in partially purified extracts of *E. coli* was stimulated by sulfhydryl compounds, and occasionally by DPN, these cofactors were added to enhance synthesis. The incubation mixture contained 10 ml. of cell-free *E. coli* extract (crude extract) (15 mg. of protein per ml.), 254 μmoles of ribose 5-phosphate, containing 5.8×10^5 c.p.m., 8 μmoles DPN, 140 μmoles of mercaptoethanol, 2500 μmoles of triethanolamine buffer, pH 8.2, in a total volume of 42 ml. After the reaction proceeded for 1 hour at 37°, the incubation mixture was placed on a Dowex 1-Cl^- column, 2.2×30 cm. and eluted with 0.0075 N KCl-0.015 N HCl; 6-ml. fractions were collected. A tenth of a milliliter of each fraction was assayed for C^{14} and color in the thiobarbituric acid test. Fig. 2 shows the data. The fractions designated as Peak IV, containing Compound I, were pooled, brought to pH 6.5 with sodium hydroxide and treated with 0.6 ml. of acid-washed activated charcoal (80 mg. of charcoal per ml.) to remove nucleotide impurities. After removal of the charcoal, the solution was concentrated in a vacuum to 3.0 ml. This solution contained 51,000 c.p.m. or about 9 per cent of the radioactivity of the ribose 5-phosphate in the original incubation mixture. If one atom of C^{14} from ribose 5-phosphate had been incorporated into the new compound, this solution of C^{14}-Compound I would have a concentration of about 8 μmoles per ml. This value is in agreement with the analytical data presented in Table I.

A large scale run to accumulate Compound I from nonlabeled ribose 5-phosphate was also carried out using a dialyzed fraction obtained after adding ammonium sulfate to the crude *E. coli* extract to secure precipitation. By this time some evidence for the additional requirement of phosphoryl-enolpyruvate as a substrate had been obtained[3] and it was used in the incubation. This incubation contained 50 ml. of a dialyzed ammonium sulfate fraction (12 mg. of protein per ml.); 75 ml. of 0.1 M triethanolamine, pH 8.2; 20 ml. of ribose 5-phosphate, 50 μmoles per ml.; 3 ml. of phosphoryl-enolpyruvate, 25 μmoles per ml.; 1.0 ml. of DPN, 40 μmoles per ml.; and 1400 μmoles of mercaptoethanol (0.10 ml.). After incubating at 37° for 1 hour, the reaction mixture was placed on a Dowex 1-Cl^- column, 3×35 cm., and eluted with 0.01 N HCl using the gradient elution technique with a mixing chamber containing 300 ml. of water. Ten-ml. fractions were collected and 0.10 ml. from each tube was tested in the thiobarbituric acid test. Compound I was eluted

[3] *Cf.* the following paper (21).

in about 4 column volumes under these conditions. The tubes containing the compound were pooled, brought to pH 6.5 with NaOH and concentrated in a vacuum to about 30 ml.; 8 ml. of acid-washed activated charcoal (80 mg. per ml.) were added to remove nucleotide impurities. After removal of the charcoal the clear supernatant fluid was concentrated to 3 ml.

Properties of Compound I—Compound I is immediately destroyed when heated with 1.0 N NaOH at 100°. In 1.0 N HCl at 100° it has a half-life of about 30 to 40 minutes as measured by the thiobarbituric acid test. Compound I shows reducing properties in the Park-Johnson test (10), but gives no color in the orcinol reaction (11) or the Dische (12) diphenylamine reaction. It contains no phosphorus. Its behavior on Dowex 1 ion exchange resins indicates that it is a weak acid. Although Compound I gives no reaction in the Friedemann-Haugen test (13) for α-keto acids, it does react with semicarbazide to give a compound absorbing at 250 mμ which is characteristic of α-keto acids (4).

Periodate oxidation of Compound I yields 2 moles of formate and 1 mole of formaldehyde per mole of Compound I (Table I). Reduction of Compound I with KBH_4 yields a compound which also forms 1 mole of formaldehyde per mole of compound when treated with periodate. The ratio of formaldehyde produced to periodate consumed is 1:3 as seen in Table II. The compound obtained from the reduction of Compound I with borohydride shows little or no color formation in the thiobarbituric acid test. The concentration of Compound I was calculated from the α-keto acid determination using pyruvic acid as the standard. These values were also checked in the thiobarbituric acid test using 2-keto-3-deoxygluconic acid as a standard, as will be discussed below.

Characterization of Compound I—On the basis of the evidence presented here, Compound I was tentatively visualized as a 2-keto-3-deoxyheptonic acid, (2-KDH) (*cf.* Compound I). The periodate oxidation data in Table I indicate that Compound I is a heptonic acid rather than a hexonic acid since, in the latter case, one would obtain only 1 mole of formate per mole of compound upon periodate treatment. In other oxidation studies, Compound I was reduced with KBH_4, lactonized in 1 N HCl and then oxidized with periodate. A 2-keto-3-deoxyhexonic acid would not be attacked by periodate after reduction and lactonization if a δ-lactone was formed (Equation 1), whereas the corresponding δ-lactone of a heptonic acid would be attacked (Equation 2).

The results of these experiments are summarized in Table III. As can be seen the lactone of the reduced form of Compound I is attacked by 1 equivalent of periodate to yield 1 equivalent of formaldehyde. A small percentage of the metasaccharinic acid lactone is oxidized by periodate under these conditions, presumably due to the presence of some γ-lactone. If the heptonic acid formed the γ-lactone, two moles of periodate would be consumed per mole of lactone. It is noteworthy that with both the 3-

TABLE II

*Oxidation of reduced Compound I with periodic acid**

Substrate	Amount	HIO_4 utilized	HCHO formed
	μmoles	*μmoles*	*μmoles*
Reduced Compound I	0.22	0.64	0.23
Reduced Compound I	0.44	1.44	0.44
Glycerol	0.20	0.40	0.40
Glycerol	0.40	0.80	0.80

* 4.4 μmoles of Compound I in 10 ml. of H_2O was reduced with 100 μmoles of KBH_4 for 30 minutes. The incubation mixture was acidified with HCl and taken to dryness seven times in the presence of methanol. The residue was dissolved in 2.0 ml. (2.2 μmoles per ml.). 0.10 ml. of this solution was treated with 2.5 μmoles of $NaIO_4$ in 1.0 ml. of 0.05 M acetate buffer, pH 5.0. Periodate consumption was followed spectrophotometrically at 260 mμ (20).

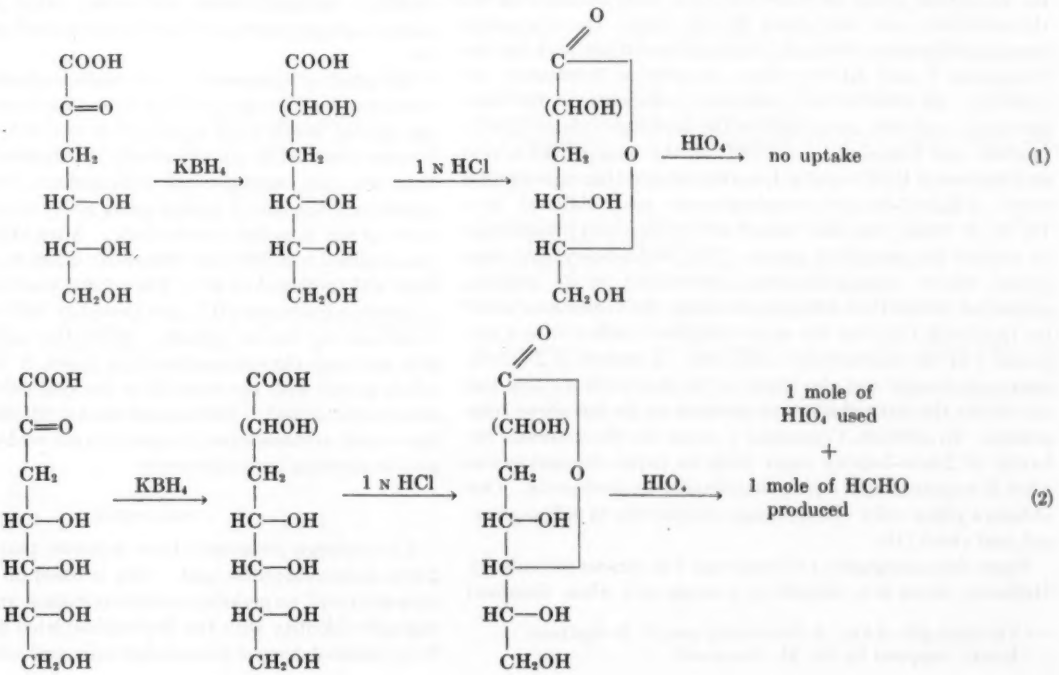


TABLE III

*Periodate oxidation of reduced, lactonized Compound I**

Compound	Amount	Periodate utilized	HCHO produced
	μmoles	*μmoles*	*μmoles*
Metasaccharinic acid lactone†	0.40	0.06	0.07
Lactone of reduced Compound I	0.28	0.32	0.26
Glycerol	0.38	0.76	0.76

* Compound I was reduced as described in Table II.

† Lactones were prepared by heating the compounds in 1 N HCl at 100° for one hour. An aliquot was oxidized with periodate as in Table II.

deoxyhexonic and -heptonic acids, the δ-lactone is preferred under the conditions of lactonization, *i.e.*, 100° for 1 hour in 1 N HCl.

The periodate oxidation of Compound I was also carried out in a Warburg respirometer at 37° to determine if any CO_2 was liberated. Under the conditions of oxidation used in the thiobarbituric acid test, there is no formation of CO_2 in the first few hours. After this time there is a slow evolution of CO_2 which ends in 24 hours. Two moles of CO_2 are formed per mole of Compound I, presumably from the further breakdown of the formyl pyruvic acid which is formed from carbon atoms 1, 2, 3, and 4 of Compound I in the initial oxidation. Concomitant with the evolution of CO_2, there is a loss of color formation in the thiobarbituric acid test. It has been reported that malonaldehyde, too, is slowly oxidized by periodate (22).

Removal of the phosphate group from the shikimic acid precursor KDPA (14) would form a 2-keto-3-deoxyheptonic acid. With this in mind, a sample of KDPA[4] was tested and observed to give the same color as Compound I in the thiobarbituric acid test. Treatment of KDPA with potato phosphatase to remove the phosphate group increases the color yield obtained in the thiobarbituric acid test about 25 per cent. The absorption spectra of the color obtained in the thiobarbituric acid test for Compound I and KDPA (after phosphatase treatment) are identical. In addition the extinction coefficients, in the thiobarbituric acid test, calculated for the dephosphorylated KDPA (40,000) and Compound I (44,000) on the basis of the α-keto acid content of KDPA and of I, are the same within experimental error. 2-Keto-3-deoxy-6-phosphogluconic acid, obtained from Dr. W. A. Wood, was also treated with potato acid phosphatase to remove the phosphate group. This dephosphorylated compound, whose concentration was determined by its reducing properties in the Park-Johnson test using the corrections noted by Doudoroff (15), has the same extinction coefficient as Compound I in the thiobarbituric acid test. A sample of 2-keto-3-deoxygalactonate[5] was also tested in the thiobarbituric acid test and shows the same absorption spectrum as do the above compounds. In addition, Compound I shows the characteristic behavior of 2-keto-3-deoxy sugar acids on paper chromatograms when it is sprayed with *o*-phenylenediamine hydrochloride. One obtains a yellow color which changes successively to yellow-green, red, and violet (16).

Paper chromatography of Compound I in various solvents (*cf.* Methods) shows it to migrate as a single spot when visualized with either silver nitrate or the semicarbazide spray. Furthermore, Compound I migrates with the same R_F as does the dephosphorylated product of KDPA in the solvent systems tested (acetone-water; ethyl acetate-pyridine-water; phenol-water).

Synthesis of 2-Keto-3-deoxyheptonic Acid—The synthesis of 2-keto-3-deoxyheptonic acid was attempted from 2-deoxy-D-glucose by a modification of the Kiliani synthesis (17): 975 mg. of 2-deoxy-D-glucose were dissolved in 10 ml. of water with 400 mg. of anhydrous calcium chloride and 325 mg. of sodium cyanide. After 24 hours at room temperature the calcium was removed by the addition of oxalic acid until a pH of 3 was obtained. After removal of the precipitate the pH of the solution was adjusted to 6 and the solution was heated at 100° for 15 minutes to hydrolyze any lactone present. This solution was concentrated in a vacuum to 2 to 3 ml. and subjected to chromatography on a cellulose column, 3 × 30 cm., with 80 per cent acetone as the eluting solvent. The main peak, appearing in 2 to 5 column volumes, was concentrated in a vacuum to a syrup (3-deoxyheptonic acid).

The 3-deoxyheptonic acid prepared synthetically from 2-deoxy-D-glucose was oxidized with potassium chlorate and vanadium pentoxide as described by De Ley and Doudoroff (16) to form, as one of the products, 2-keto-3-deoxyheptonic acid. After the oxidation of the heptonic acid (1 mmole) was complete, the mixture was chromatographed on a cellulose column, 3 × 33 cm., with 85 per cent acetone as the eluting solvent. Two peaks, which gave the characteristic color in the thiobarbituric acid test with a maximal absorption at 545 to 550 mμ, were isolated. The earliest peak, appearing in about 1.5 to 2 column volumes, and the later peak, appearing in 2.5 to 3.5 column volumes, were collected and separately concentrated in a vacuum. The early peak was rechromatographed on a cellulose column as above, and the peak was reisolated. This rechromatographed early peak was found to give one spot on paper chromatography, as visualized with either silver nitrate or the semicarbazide reagent, and moved with the same R_F as Compound I in these solvent systems: methanol-formic acid-water; ethyl acetate-pyridine-water; acetone-water; and methanol-ammonium hydroxide-water.

Reduction of Compound I with Sodium Borohydride—The reduction of the keto group of Compound I to form the corresponding alcohol should yield a mixture of two 3-deoxyheptonic acid isomers identical to the chemically synthesized product. This reduction was accomplished with sodium borohydride. Ten μmoles of Compound I in 0.25 ml. of water at pH 6 were treated with 1.0 mg. of sodium borohydride. After 18 hours the solution was acidified with HCl and repeatedly taken to dryness in a vacuum with methanol at 50°. The residue was taken up in 1.0 ml. of water, adjusted to pH 7, and heated at 100° for 10 minutes to hydrolyze any lactone present. When the material obtained in this way was chromatographed on paper, it showed one spot which moved with the same R_F as the synthetically prepared 3-deoxyheptonic acid. The solvents used were: ethyl acetate-pyridine-water; acetone-water; propanol-formic acid-water; and methanol-ammonium hydroxide-water.

DISCUSSION

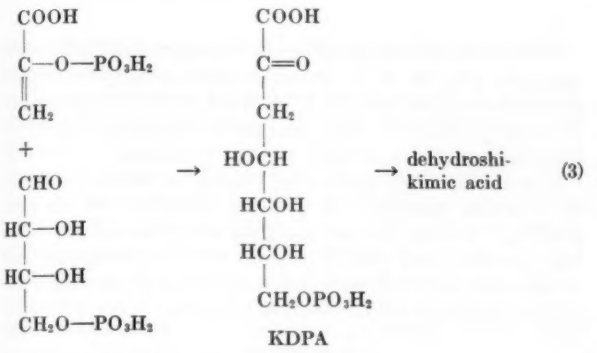
The evidence presented above indicates that Compound I is 2-keto-3-deoxyheptonic acid. This is based on its properties as an α-keto acid, on periodate oxidation studies, and on its chromatographic identity with the dephosphorylated product obtained from 2-keto-3-deoxy-7-phosphoheptonic acid reported by Roth-

[4] The kind gift of Drs. J. Rothschild and D. B. Sprinson.

[5] Kindly supplied by Dr. M. Doudoroff.

schild (23). Compound I also exhibits typical color reactions observed with other 2-keto-3-deoxy sugar acids.

The formation of KDA in crude cell-free extracts of *E. coli* strain B, when ribose 5-phosphate is the substrate, is an important pathway for pentose metabolism. Up to 20 per cent of the ribose 5-phosphate initially present in the incubation can be converted to KDA within an hour under the conditions used. The isolation and accumulation of KDA *in vitro*, as reported here, represents the first direct demonstration of this compound in biological systems. The occurrence of KDA strongly supports the mechanism first investigated by Srinivasan and his coworkers (14) for the formation of dehydroshikimic acid from erythrose 4-phosphate and phosphoryl-enolpyruvate. 2-Keto-3-deoxy-phosphoheptonic acid was postulated as an intermediate in this reaction, as shown in Equation 3.

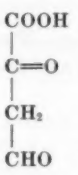


(3)

Alternately, removal of the phosphate group from KDPA would yield a 2-keto-3-deoxyheptonic acid with carbon atoms 4, 5, and 6 of the D-glucose configuration which is presumably Compound I.

Since erythrose 4-phosphate and phosphoryl-enolpyruvate could easily be formed from ribose 5-phosphate by known reactions (18), this pathway seems the likely biosynthetic route to KDA. Further evidence in support of this theory is presented in the following paper (21).

The thiobarbituric acid test, originally developed for 2-deoxyribose by Waravdekar and Saslaw, provides an excellent and very sensitive method for the determination of compounds which can form formyl pyruvic acid (Compound II) upon periodate oxidation. The thiobarbituric acid test, when modified as described in this paper, seems quite specific for this grouping. No other compounds tested, except the 2-keto-3-deoxy sugar acids, react in the thiobarbituric acid test to form a chromogen whose absorption maximum is at 545 to 550 mμ. As expected, reduction of the 2-keto group with borohydride prevents color formation in the thiobarbituric acid test.



COMPOUND II

SUMMARY

1. The formation and accumulation of 2-keto-3-deoxy heptonic acid is observed in crude cell-free extracts of *Escherichia coli* strain B incubated with ribose 5-phosphate.

2. A modification of the color test developed by Waravdekar and Saslaw for deoxy sugars has been found to provide a sensitive means for detecting and measuring sugar acids containing a 2-keto-3-deoxy grouping.

REFERENCES

1. WARAVDEKAR, V. S., AND SASLAW, L. D., *Biochim. et Biophys. Acta*, **24**, 439 (1957).
2. NOSSAL, P. M., *Australian J. Exptl. Biol. Med. Sci.*, **31**, 583 (1953).
3. BÜCHER, T., *Biochim. et Biophys. Acta*, **1**, 292 (1947).
4. MACGEE, J., AND DOUDOROFF, M., *J. Biol. Chem.*, **210**, 617 (1954).
5. MACFADYEN, D. A., *J. Biol. Chem.*, **158**, 107 (1945).
6. RABINOWITZ, J. C., AND PRICER, W. E., JR., *J. Biol. Chem.*, **229**, 321 (1957).
7. HESTRIN, S., *J. Biol. Chem.*, **180**, 249 (1949).
8. ANET, E. F. L. T., AND REYNOLD, T. M., *Nature*, **174**, 930 (1954).
9. HORECKER, B. L., GIBBS, M., KLENOW, H., AND SMYRNIOTIS, P. Z., *J. Biol. Chem.*, **207**, 393 (1954).
10. PARK, J. T., AND JOHNSON, M. J., *J. Biol. Chem.*, **181**, 149 (1949).
11. MEJBAUM, W., *Z. physiol. Chem.*, **258**, 117 (1939).
12. DISCHE, Z., *Mikrochemie*, **8**, 4 (1930).
13. FRIEDEMANN, T. E., AND HAUGEN, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
14. SRINIVASAN, P. R., KATAGIRI, M., AND SPRINSON, D. B., *J. Am. Chem. Soc.*, **77**, 4943 (1955).
15. PALLERONI, N. J., AND DOUDOROFF, M., *J. Biol. Chem.*, **223**, 499 (1956).
16. DE LEY, J., AND DOUDOROFF, M., *J. Biol. Chem.*, **227**, 745 (1957).
17. HUDSON, C. S., HARTLEY, O., AND PURVES, C. B., *J. Am. Chem. Soc.*, **56**, 1248 (1934).
18. HORECKER, B. L., *Brewers Digest*, **28**, 214 (1953).
19. SAKAMI, W., *J. Biol. Chem.*, **187**, 369 (1950).
20. HORECKER, B. L., HURWITZ, J., AND WEISSBACH, A., *J. Biol. Chem.*, **218**, 785 (1956).
21. HURWITZ, J., AND WEISSBACH, A., *J. Biol. Chem.*, **234**, 710 (1959).
22. BOBBITT, J. M., *Advances in Carbohydrate Chem.*, **11**, 4 (1956).
23. ROTHSCHILD, J., *Federation Proc.*, **16**, 239 (1957).

The Formation of 2-Keto-3-deoxyheptonic Acid in Extracts of *Escherichia coli* B

II. ENZYMIC STUDIES*

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In the preceding paper (1), evidence concerning the proof of structure of 2-keto-3-deoxyheptonic acid was presented and it was indicated that this compound is a major product of ribose 5-phosphate metabolism in extracts of *Escherichia coli* strain B. This report is concerned with the enzymic synthesis of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* strain B and is the first direct observation of the biosynthesis of this compound. It is suggested that 2-keto-3-deoxyheptonic acid arises from erythrose 4-phosphate and phosphoryl-enolpyruvate. The condensation product, presumed to be 2-keto-3-deoxy-7-phosphoheptonic acid is then dephosphorylated, yielding 2-keto-3-deoxyheptonic acid. This pathway of 2-keto-3-deoxyheptonic acid synthesis is in accord with the observations reported by Srinivasan *et al.* (2) on the biosynthesis of 2-keto-3-deoxy-7-phosphoheptonic acid which has been demonstrated as an intermediate in the formation of shikimic acid.

EXPERIMENTAL

The following materials were obtained from commercial sources: crystalline ATP, ADP, AMP, and DPNH (Sigma Chemical Company), ribose 5-phosphate (Schwarz Laboratories) and P-enolpyruvate (California Foundation for Biochemical Research). The latter compound was obtained as the tricyclohexylamine salt and was converted to the potassium salt after passage through Dowex 50-K^+ resin. Streptomycin sulfate was obtained from Merck and Company.

Pentose was determined by the method of Mejbaum (3), and sedoheptulose was measured as described by Horecker *et al.* (4). P_i was determined by the method of Fiske and SubbaRow (5). KDA[1] was determined essentially by the procedure described by Waravdekar and Saslaw (6) as described in the preceding paper (1). Protein was measured by the method of Sutherland *et al.* (7).

P-enolpyruvate was measured with phosphoryl-enolpyruvic kinase and lactic dehydrogenase (8). The latter two enzymes were obtained from Worthington Biochemical Corporation as the twice crystallized lactic dehydrogenase.

Sedoheptulose 1,7-diphosphate and sedoheptulose 7-phosphate were gifts from Dr. B. L. Horecker; 2-keto-3-deoxy-6-phosphogluconate was a gift from Dr. W. A. Wood; KDPA was generously provided by Drs. P. R. Srinivasan and D. B. Sprinson, and erythrose 4-phosphate was a gift from Dr. C. Ballou.

E. coli strain B was grown with shaking for 16 hours at 30° in the following medium: 1.09 per cent K_2HPO_4, 0.85 per cent KH_2PO_4, 1 per cent glucose, and 1 per cent yeast extract. The cells were harvested after 12 hours at 30° by centrifugation and washed twice with cold water and stored in the frozen state without loss of activity over a period of 3 months.

RESULTS

The incubation mixture (0.3 ml.) used for the formation of 2-KDA contained 20 μmoles of glycylglycine buffer, pH 7.5; 4 μmoles of $MgCl_2$, 2 μmoles of ribose 5-phosphate, 0.6 μmole of the P-enolpyruvate, 0.1 μmole of mercaptoethanol, and varying amounts of enzyme. The time of incubation was 30 minutes at 38°, unless otherwise stated.

The enzyme preparation was made as follows: 25 gm. of *E. coli* strain B, suspended in 100 ml. of 0.01 M glycylglycine buffer, pH 7.4, were exposed for 15 minutes in a 10-kc. Raytheon sonic oscillator and then centrifuged for 30 minutes at 10,000 × *g*. at 0°. The supernatant fluid (crude extract) was then diluted with an equal volume of distilled water (total volume, 200 ml.) and 30 ml. of 5 per cent streptomycin sulfate was added with stirring. After 10 minutes, the suspension was centrifuged and the clear supernatant fluid (streptomycin sulfate supernatant, volume 210 ml.) was adjusted to pH 5.0 by the addition of 2 ml. of 2 M acetate buffer, pH 5.0. After 10 minutes, the precipitate was removed by centrifugation (pH 5.0 fraction). The temperature of the clear supernatant fluid (215 ml.) was adjusted to about 2° and then treated with 41 ml. of acetone (−10°) and the precipitate collected (Acetone I). The supernatant fluid (250 ml.) was treated with 50 ml. more acetone and the precipitate collected (Acetone II). All fractions were dissolved in 10 ml. of 0.1 M glycylglycine buffer, pH 7.4. The activites obtained with the above fractions are summarized in Table I. As shown, this purification procedure indicated that more than one fraction was required for KDA formation. While there was demonstrable activity with either acetone fraction alone, the combination of Acetone I and Acetone II fractions resulted in nearly 10-fold increase over the sum of their individual activities. While the pH 5.0 Fraction augmented this activity, this fraction did not appear

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[1] The abbreviations used are: KDA, 2-keto-3-deoxyheptonic acid; KDPA, 2-keto-3-deoxy-7-phosphoheptonic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

TABLE I

Preparation of enzyme fractions for KDA synthesis

Fraction	Total units*	Protein
		mg./ml.
Crude extract	144	20
Streptomycin sulfate supernatant	180	4.0
pH 5.0 Fraction	0	4.8
Acetone I	4.4	6.0
Acetone II	14.8	8.0
pH 5.0 + Acetone I + Acetone II	160	
Acetone I + Acetone II	128	
pH 5.0 + Acetone I	10	
pH 5.0 + Acetone II	35	

* Total units refers to μmoles of KDA formed per hour with the conditions described under Results.

to be required provided the two acetone fractions were simultaneously employed.

With crude extracts, under these conditions, both P_i and DPN stimulated KDA production. ATP had very little effect on this synthesis, while ADP addition markedly inhibited KDA synthesis. As shown in Table II, P-enolpyruvate addition stimulated KDA formation. In addition when P-enolpyruvate was added to the acetone fractions, P_i and DPN additions were inhibitory. Also shown in Table II, hexose diphosphate does not replace ribose 5-phosphate, nor does pyruvate replace P-enolpyruvate. On the basis of the results shown in Table II, it was concluded that 2-KDA synthesis was dependent on both ribose 5-phosphate and P-enolpyruvate addition.

Incubation of ribose 5-phosphate with the enzyme fraction Acetone I resulted in a decrease in pentose as measured in the orcinol test (4); evidence of sedoheptulose formation was obtained. The latter observation suggested the presence of the enzyme transketolase (9). A number of compounds which are substrates of this enzyme were compared with ribose 5-phosphate as precursors of KDA. In the presence of triose phosphate (generated by the addition of hexose diphosphate and aldolase) and P-enolpyruvate, sedoheptulose 7-phosphate and fructose 6-phosphate addition resulted in demonstrable KDA formation. However, when sedoheptulose diphosphate or hexose diphosphate was used in place of ribose 5-phosphate, KDA was not produced.

Once the structure of KDA was ascertained (1), it was evident that the synthesis resembled that found by Srinivasan *et al.* (2) for KDPA. These workers have observed that erythrose 4-phosphate plus P-enolpyruvate were almost quantitatively converted to 5-dehydroshikimic acid by cell-free extracts of *E. coli*. In view of these observations, erythrose 4-phosphate was tested as a precursor of 2-KDH and these results are presented in Table III. It is clear that erythrose 4-phosphate is a much more effective precursor than ribose 5-phosphate. In addition, the condensation reaction between erythrose 4-phosphate and P-enolpyruvate appeared to be catalyzed only by the enzyme fraction Acetone II since the addition of the Acetone I fraction had no effect on the reaction.

As discussed in the previous paper (1), the product, KDA, was devoid of phosphate. Since the condensation product obtained from erythrose 4-phosphate and P-enolpyruvate would be expected to contain this substance, its absence suggested the presence of a phosphatase-like activity, and this was found to be the case. As shown in Table IV, KDPA was readily dephos-

TABLE II

Requirements for KDA formation

The following conditions were used: In Experiment No. 1, the reaction mixture (0.3 ml.) contained 2 μmoles of ribose 5-phosphate, 20 μmoles of glycylglycine buffer, pH 7.5, 0.1 μmole of mercaptoethanol, 4 μmoles of $MgCl_2$, 0.6 μmoles of P-enolpyruvate, 1 μmole of DPN, 10 μmoles of potassium phosphate buffer, pH 7.5, 48 μg. of pH 5.0 fraction, 60 μg. of Acetone I and 80 μg. of Acetone II. In Experiment No. 2, the additions were the same as in Experiment No. 1 with the exception that P_i and DPN were omitted.

Experiment No.	Additions	2-KDH formed in 30 minutes
		mμmoles
1	pH 5.0 Fraction + Acetone I + Acetone II	61
	Omit P-enolpyruvate	34
	Acetone I + Acetone II	52
	Acetone I + Acetone II; omit P-enolpyruvate	18
	Acetone I + Acetone II; omit P_i	58
	Acetone I + Acetone II; omit P_i and DPN	85
2	Acetone I + Acetone II	85
	Acetone I + Acetone II + P_i and DPN	46
	Omit ribose 5-phosphate	0
	Omit P-enolpyruvate	5
	Pyruvate in place of P-enolpyruvate	0
	Hexose diphosphate; omit ribose 5-phosphate	11

TABLE III

KDA synthesis from erythrose 4-posphate

The conditions used were as described in Table II, Experiment No. 2.

Substrate added	2-KDH formed in 30 minutes
	mμmoles
Ribose 5-phosphate (2 μmoles)	51
Erythrose 4-phosphate (0.3 μmole)	80
Erythrose 4-phosphate (0.6 μmole)	118

TABLE IV

Formation of P_i from various substrates

The incubation mixture (0.3 ml.) contained the substrate as indicated, 20 μmoles of Tris buffer, pH 7.5, 4 μmoles of $MgCl_2$, 60 μg. of Acetone I fraction and 80 μg. of Acetone II fraction. The reaction was terminated by the addition of 0.1 ml. of 25 per cent trichloroacetic acid and P_i estimated by the method of Fiske and SubbaRow (5).

Substrate added	P_i formed in 30 minutes
	μmoles
KDPA (1 μmole)	0.23
2-keto 3-deoxy 6-phosphogluconate (1 μmole)	0.26
Ribose 5-phosphate (2 μmoles)	0.05
Fructose 6-phosphate (2 μmoles)	0.05

TABLE V

Stoichiometry of KDA System

All values are expressed in μmoles. In Experiment No. 1, the incubation mixture (0.3 ml.) contained 2 μmoles of ribose 5-phosphate, 0.1 μmole of mercaptoethanol, 4 μmoles of $MgCl_2$, 20 μmoles of glycylglycine buffer, pH 7.5, P-enolpyruvate as indicated, 0.12 mg. of Acetone I fraction and 0.16 mg. of Acetone II fraction. The incubation period was as indicated, after which time the reaction was terminated by the addition of cold 1 N $HClO_4$ (0.1 ml.), followed by 0.4 ml. of water. The solution was neutralized with KOH and the $KClO_4$ removed by centrifugation in the cold. P-enolpyruvate, P_i and KDA were determined as previously described. In Experiment No. 2, ribose 5-phosphate was replaced with 0.4 μmoles of erythrose 4-phosphate. All other additions were the same as above.

Experiment No.	Time of incubation	P-enolpyruvate	KDA	P_i
	minutes	*μmoles*	*μmoles*	*μmoles*
1	0	0.58	0	0.02
	20	0.48	0.14	0.26
	30	0.31	0.21	0.38
2	0	0.42	0	0.06
	40	0.17	0.23	0.55

phorylated by the enzyme fractions used. In addition, this phosphatase-like activity appeared to be about five times more rapid on this compound than on ribose 5-phosphate or fructose 6-phosphate.

The over-all stoichiometry of the reactions involved is summarized in Table V. The balance found is in agreement with the over-all Equation 1.

$$\text{Erythrose 4-phosphate} + \text{P-enolpyruvate} \rightarrow \text{KDA} + 2\ P_i \quad (1)$$

The relationship, 1 mole of P-enolpyruvate disappearing:1 mole of 2-KDH formation:2 moles of P_i formation, was also approximated with ribose 5-phosphate as substrate. It was also noted that on prolonged incubation (60 to 120 minutes) with ribose 5-phosphate as substrate, more P-enolpyruvate disappeared than could be accounted for in the above relationship. The nature of this disappearance was not further investigated.

DISCUSSION

The data presented above are in accord with the results obtained by Srinivasan *et al.* (2). KDA, as measured colorimetrically (6), does not disappear when incubated with the above acetone fractions. However, in the presence of crude extracts of *E. coli*, the compound is converted to a form which no longer reacts colorimetrically. Thus, in the presence of 0.75 mg. of protein (crude extract), 25 mμmoles of KDA disappeared (50 per cent of the substrate added) in 15 minutes. The product of this reaction was not determined. However, KDA is not a precursor in dehydroquinic acid formation in the system used by Sprinson *et al.*[2]

The stoichiometry presented, as well as the presence of a dephosphorylating system, would suggest that the pathway of synthesis of KDA is shown by Equation 2.

$$\text{P-enolpyruvate} + \text{erythrose 4-phosphate} \rightarrow \text{KDPA} + P_i \quad (2)$$
$$\text{KDPA} \rightarrow \text{KDA} + P_i$$

SUMMARY

1. In the presence of ribose 5-phosphate or erythrose 4-phosphate and phosphoenolpyruvate, enzyme fractions have been obtained from *E. coli* which catalyze the formation of 2-keto-3-deoxyheptonic acid.

2. These enzyme fractions also contain a phosphatase activity which yields inorganic phosphate from 2-keto-3-deoxy-7-phosphoheptonic acid, as well as 2-keto-3-deoxy-6-phosphogluconate. Ribose 5-phosphate and fructose 6-phosphate are slowly dephosphorylated by these preparations.

3. The over-all balance is in accord with the reaction sequence: erythrose 4-phosphate + phosphoenolpyruvate → 2-keto-3-deoxy-7-phosphoheptonic acid + inorganic phosphate → 2-keto-3-deoxyheptonic acid + inorganic phosphate.

REFERENCES

1. WEISSBACH, A., AND HURWITZ, J., *J. Biol. Chem.*, **234**, 705 (1959).
2. SRINIVASAN, P. R., KATAGIRI, M., AND SPRINSON, D. B., *J. Am. Chem. Soc.*, **77**, 4943 (1955).
3. MEJBAUM, N., *Z. physiol. Chem.*, **258**, 117 (1939).
4. HORECKER, B. L., SMYRNIOTIS, P. Z., AND KLENOW, H., *J. Biol. Chem.*, **205**, 661 (1953).
5. FISKE, C. H., AND SUBBAROW, Y., *J. Biol. Chem.*, **66**, 375 (1925).
6. WARAVDEKAR, V. S., AND SASLAW, L. D., *Biochim. et Biophys. Acta*, **24**, 439 (1957).
7. SUTHERLAND, E. W., CORI, C. F., HAYNES, R., AND OLSEN, N. S., *J. Biol. Chem.*, **180**, 825 (1949).
8. KORNBERG, A., AND PRICER, W. E., JR., *J. Biol. Chem.*, **193**, 481 (1951).
9. HORECKER, B. L., *Brewers Digest*, **28**, 214 (1953).

[2] Personal communication from Dr. D. B. Sprinson.

The Conversion of Phosphoenolpyruvic Acid and D-Erythrose 4-Phosphate to 5-Dehydroquinic Acid*

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It was shown in previous work that cell-free extracts of *Escherichia coli* converted sedoheptulose 1,7-diphosphate nearly quantitatively to 5-dehydroquinic acid (1, 2).[1] Although carbon atoms 4, 5, 6, and 7 of SDP[2] were shown to be incorporated, probably as a unit, into positions 3 to 6 of the dehydroquinate, tracer studies on the biosynthesis of shikimic acid (3) indicated that the utilization of SDP involved a cleavage of the molecule rather than a direct transformation of the intact 7-carbon chain to an acyclic precursor of dehydroquinate (2). Further studies, to be reported here, have shown that the pathway between SDP and dehydroquinate involves the cleavage of SDP to D-erythrose 4-phosphate (erythrose-4-P) and dihydroxyacetone phosphate, conversion of dihydroxyacetone phosphate to phosphoenolpyruvate (P-enolpyruvate), and synthesis of 5-dehydroquinate from erythrose-4-P and P-enolpyruvate. A part of these results appeared in preliminary form (4).

EXPERIMENTAL

The preparation of cell-free extracts of *E. coli* mutant 83-24 (blocked after shikimic acid) (5), and the assay for 5-dehydroquinic acid and 5-dehydroshikimic acid, were described previously (1).[3] The KDHP used in the experiments of Table V was prepared by an unpublished method;[4] the preparation of the acetone fraction (KDHP synthetase), and the determination of KDHP are described in the following paper (8).

The mutant strains used in this work were kindly furnished by Professor B. D. Davis. The cyclohexylammonium erythrose-4-P dimethylacetal (9) was a generous gift of Professor C. E. Ballou. Erythrose-4-P was prepared as sodium salt (9). Barium P-enolpyruvate was a generous gift of Mr. W. E. Pricer, Jr., and was converted to the potassium salt. SDP was prepared enzymatically (10) through the kindness of Professor B. L. Horecker. Crystalline muscle aldolase and a sample of SDP which was assayed (11) to be enzymatically pure were gifts of Professor E. Racker.

The following compounds were obtained from commercial sources: fructose 1,6-diphosphate and D-3-phosphoglyceric acid (3-P-glyceric acid), Schwarz Laboratories, Inc.; DPN, Pabst Laboratories; and crystalline sodium ATP, Sigma Chemical Company.

Protein concentration was estimated spectrophotometrically (12).

RESULTS AND DISCUSSION

Conversion of Erythrose-4-P and P-enolpyruvate to 5-dehydroquinate—As shown previously (2), SDP was nearly quantitatively converted to 5-dehydroquinate by extracts of *E. coli* in a 2-hour incubation. It can be seen from Table I that this conversion was completely blocked by fluoride and iodoacetate. In the presence of fluoride, synthesis was restored by P-enolpyruvate, while in the presence of iodoacetate either this substance or 3-phosphoglyceric acid (3-P-glyceric acid), but not DPN, restored synthesis. These results suggested that the glycolytic reactions from triose phosphate to P-enolpyruvate are involved in the conversion of SDP to 5-dehydroquinate. The suppression of 5-dehydroquinate formation by fluoride and iodoacetate would be due to their well known inhibition of enolase and triose phosphate dehydrogenase, respectively. A reasonable explanation of the results in Table I can be based on the reaction sequences 1 to 3 for the conversion of SDP to 5-dehydroquinate.

$$\text{SDP} \rightleftharpoons \text{erythrose-4-P} + \text{dihydroxyacetone phosphate (10)} \quad (1)$$

$$\text{Dihydroxyacetone phosphate} \rightleftharpoons \text{3-P-glyceric acid} \rightleftharpoons \text{P-enolpyruvate} \quad (2)$$

$$\text{Erythrose-4-P} + \text{P-enolpyruvate} \rightarrow \rightarrow \text{5-dehydroquinate} \quad (3)$$

It was possible to put this postulated mechanism to the test when erythrose-4-P became available through the elegant work of Ballou *et al.* (9). As can be seen from Table II, P-enolpyruvate and erythrose-4-P were converted nearly quantitatively to dehydroquinate at a more rapid rate than SDP. Neither fluoride nor iodoacetate were able to block the conversion of P-enolpyruvate and erythrose-4-P. Pyruvate could replace P-enolpyruvate to a limited extent, presumably as a result of the action of ATP-P-enolpyruvate transphosphorylase, or of the following sequence (14).

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[1] Under the experimental conditions described previously (1, 2), and in the present work, 5-dehydroshikimic acid is the actual end product of this conversion. The reactions of interest here, however, are those concerned with the formation of 5-dehydroquinic acid, the first known cyclic intermediate in aromatic biosynthesis. This intermediate will therefore be referred to as the end product of the reactions discussed in this paper.

[2] The abbreviations used are: SDP, sedoheptulose 1,7-diphosphate; KDHP; 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate.

[3] The mutant used for the bioassay is a quintuple aromatic auxotroph, *Aerobacter aerogenes* strain A170-143SI (6), which responds equally well to 5-dehydroquinic, 5-dehydroshikimic, and shikimic acids.

[4] D. B. Sprinson and J. Rothschild, in preparation (*cf.* (7)).

TABLE I

Effect of fluoride and iodoacetate on conversion of SDP to 5-dehydroquinic acid

The incubation mixture contained 0.1 ml. of extract (2 mg. of protein), 5 μmoles of $MgCl_2$,* 50 μmoles of potassium phosphate buffer, pH 7.4, and 0.25 μmole of potassium SDP† in a final volume of 1 ml. Other additions, when present, were 10 μmoles of KF or 0.5 μmole of iodoacetate. When iodoacetate was added, the solution, 0.95 ml., was preincubated at 37° for 15 minutes before the addition of SDP. After incubation at 37° for the indicated length of time aliquots were removed for bioassay (1).

Substrate and additions	Conversion to dehydroquinate	
	1 hour	2 hours
	%	%
SDP	39	83
SDP + fluoride	0	0
SDP + fluoride + 0.5 μmole of FDP‡	0	0
SDP + fluoride + 0.5 μmole of PGA	0	0
SDP + fluoride + 0.5 μmole of pyruvate	0	0
SDP + fluoride + 0.3 μmole of PEP	37	80
SDP + iodoacetate	0	0
SDP + iodoacetate + 0.25 μmole of DPN	0	0
SDP + iodoacetate + 0.5 μmole of FDP	0	0
SDP + iodoacetate + 0.5 μmole of PGA	46	83
SDP + iodoacetate + 0.5 μmole of pyruvate	0	0
SDP + iodoacetate + 0.3 μmole of PEP	46	83

* Mg^{++} is not needed in the conversion of erythrose-4-P and P-enolpyruvate to dehydroquinate, but is required in reactions involved in the formation of P-enolpyruvate from triose phosphate.

† As estimated by the orcinol reaction (13) on a sample contaminated with fructose-1,6-di-P.

‡ FDP, fructose-1,6-di-P; PGA, 3-P-glyceric acid; PEP, P-enolpyruvate.

TABLE II

Synthesis of dehydroquinate from D-erythrose-4-phosphate and phosphoenolpyruvate

The incubation and assay procedures were as in Table I. SDP and erythrose-4-P were used in 0.25-μmole amounts.

Substrates and additions	Conversion to dehydroquinate	
	1 hour	2 hours
	%	%
SDP	39	83
Erythrose-4-P	0	0
0.3 μmoles of PEP*	0	0
Erythrose-4-P + 0.3 μmole of PEP	88	86
Erythrose-4-P + fluoride + 0.3 μmole of PEP	88	88
Erythrose-4-P + fluoride + 0.5 μmole of PGA	0	0
Erythrose-4-P + iodoacetate + 0.3 μmole of PEP	90	90
Erythrose-4-P + iodoacetate + 0.5 μmole of PGA	90	90
Erythrose-4-P + 0.5 μmole of pyruvate		12
Erythrose-4-P + 0.5 μmole of pyruvate + 0.5 μmole of ATP		12

* PEP, P-enolpyruvate; PGA, 3-P-glyceric acid.

$$\begin{aligned} \text{Pyruvate} + \text{TPNH} + H^+ + CO_2 &\rightarrow \text{malate} + \text{TPN}^+ \\ \text{Malate} + \text{DPN}^+ &\rightarrow \text{oxaloacetate} + \text{DPNH} + H^+ \\ \text{Oxaloacetate} + \text{ITP} &\rightarrow \text{P-enolpyruvate} + CO_2 + \text{IDP} \\ \text{IDP} + \text{ATP} &\rightarrow \text{ITP} + \text{ADP} \end{aligned} \quad (4)$$

It must be assumed that most of the added pyruvate was oxidized and that some of the ATP, formed through oxidative phosphorylation, was used for P-enolpyruvate synthesis. Added ATP did not stimulate pyruvate utilization.[5]

These results lend strong support to previous conclusions, based on tracer studies, that in the biosynthesis of shikimic acid from glucose the carboxyl and carbon atoms 1 and 2 are derived from a 3-carbon intermediate of glycolysis, and carbons 3, 4, 5, and 6 are derived from tetrose phosphate, generated via the pentose phosphate pathway (3). It was further suggested that the carbon atom corresponding to carbon 3 of triose phosphate became attached to carbon 1 of the tetrose. This is in accordance with the reasonable expectation that carbon 3 of P-enolpyruvate is condensed with carbon 1 of erythrose-4-P in the first step of the reactions indicated in Equation 3. Further evidence on this point is reported in the following paper (8) where P-enolpyruvate and erythrose-4-P are shown to condense to form KDHP and inorganic phosphate.

5-Dehydroquinate Formation in Charcoal-treated Extracts—The oxidation of triose phosphate to 3-P-glyceric acid (Equation 2) requires DPN, ADP, and inorganic phosphate. Presumably the crude extracts used in this study contained the catalytic amounts of DPN and ADP needed to form 3-P-glyceric acid which was further converted to P-enolpyruvate. Regeneration of DPN would take place through the action of DPNH oxidase (which is known to be present in *E. coli* extracts (15)) while regeneration of ADP would be expected as a result of ATPase activity. It was shown previously (2) that treatment of the extracts with charcoal led to a loss of activity in the conversion of SDP to dehydroquinate, and that the addition of DPN alone was sufficient to restore it.[6] As would be expected erythrose-4-P and P-enolpyruvate were readily converted to dehydroquinate without the addition of DPN (Table III). In the presence of P-enolpyruvate the charcoal-treated extracts were able to utilize SDP as a source of erythrose-4-P for dehydroquinate formation, although the yields at 1 hour were lower than from SDP plus DPN.

The decreased rate of synthesis from SDP and P-enolpyruvate in the charcoal-treated extracts may be explained by the observation (8) that SDP inhibits the condensation of erythrose-4-P and P-enolpyruvate. The effect of this inhibition on dehydroquinate formation from SDP is shown in Table IV. Concentrations above 0.25 μmoles per ml. are progressively more inhibitory. The earlier finding (4) that in charcoal-treated extracts SDP was unable to serve as a source of erythrose-4-P in the presence of P-enolpyruvate was due to a contamination[7] of the SDP by

[5] ATP was inhibitory in the conversion of erythrose-4-P and P-enolpyruvate to 5-dehydroquinic acid. At a concentration of 10^{-3} M ATP (incubation mixture as described in Table II) the yield of dehydroquinate was decreased by 45 per cent.

[6] It must therefore be assumed that not all of the ADP was removed by the charcoal treatment, or that 1,3-diphosphoglycerate was degraded in a way not involving ATP-phosphoglyceric transphosphorylase. Subsequent work (16) showed that the immediate product of the P-enolpyruvate condensation with erythrose-4-P, *i.e.* KDHP, also requires DPN for conversion to 5-dehydroquinic acid. A more thorough treatment with charcoal (6 to 7 mg. per mg. of protein with gentle shaking for 1 hour) is necessary to show this requirement.

[7] We are grateful to Professor E. Racker for the information that this sample of SDP contained 23 per cent of fructose-1,6-di-P.

TABLE III

DPN requirement in conversion of SDP to dehydroquinate

For the removal of DPN, extracts containing 20 mg. of protein per ml. were allowed to stand with charcoal (1 mg. per mg. of protein) at 0°. The mixture was stirred every 5 minutes by gentle aspiration into and release from a pipette. After 30 minutes the charcoal was removed by centrifugation at 0°. The reaction mixtures contained 0.25 μmole of erythrose-4-P, SDP, or DPN, and 0.3 μmole of PEP. The incubation and assay procedures were as in Table I.

Substrate and additions	Conversion to dehydroquinate	
	1 hour	2 hours
	%	%
Erythrose-4-P + PEP	80	90
SDP*	0	0
SDP* + DPN	68	68
SDP* + PEP†	28	68
SDP* + PEP + 0.1 μmole of FDP	18	33
SDP* + PEP + 0.2 μmole of FDP	14	28
SDP* + PEP + 0.4 μmole of FDP	9	24
SDP‡ (used in previous work (4)) + PEP	±	16
SDP‡ + DPN	58	75

* Pure SDP, by enzymatic assay.

† PEP, P-enolpyruvate; FDP, fructose-1,6-di-P.

‡ Crude SDP (*cf.* second footnote to Table I).

fructose-1,6-di-P, which is also an inhibitor of the erythrose-4-P and P-enolpyruvate condensation (8). The inhibitory effect of fructose-1,6-di-P on the formation of dehydroquinate from SDP and P-enolpyruvate in charcoal treated extracts is shown in Table III. It is noteworthy that fructose-1,6-di-P is a more pronounced inhibitor of the conversion of SDP plus P-enolpyruvate to dehydroquinate (Table III) than of the P-enolpyruvate–erythrose-4-P condensation (8). This can be accounted for by the very small concentrations of erythrose-4-P present when SDP is used as a source of tetrose phosphate, since the aldolase equilibrium favors the formation of SDP (10). In the presence of DPN, both SDP and fructose-1,6-di-P are removed by glycolytic reactions.

That dehydroquinate formation from SDP involves an uncomplicated cleavage of SDP to erythrose-4-P and triose phosphate is further supported by the observations shown in Table V. When a purified fraction inactive on SDP and capable of condensing erythrose-4-P and P-enolpyruvate to KDHP (8), was supplemented with crystalline muscle aldolase, it formed KDHP also from SDP and P-enolpyruvate.

TABLE IV

Inhibitory effect of increasing concentration of SDP on its conversion to 5-dehydroquinic acid

The incubation and assay procedures were as in Table I.

SDP*	Conversion to dehydroquinate	
	1 hour	2 hours
μmoles/ml.	μmoles	μmoles
0.25	0.081	0.16
0.50	0.16	0.24
1.0	0.11	0.23
2.0	0.090	0.23
4.0	0.14	0.28

* Pure SDP, by enzymatic assay (11).

TABLE V

Conversion of SDP and P-enolpyruvate to KDHP by muscle aldolase and purified KDHP synthetase

Substrates	KDHP formed at:		
	15 minutes	30 minutes	60 minutes
	μmoles	μmoles	μmoles
Erythrose-4-P + PEP*	0.63	0.64	0.69
SDP + PEP†	0.36	0.47	0.56

* One μmole each of erythrose-4-P and P-enolpyruvate (PEP) were preincubated with 100 μmoles of potassium phosphate buffer, pH 6.4, in a volume of 1.96 ml., at 37° for 10 minutes. 0.04 ml. of acetone fraction (8) (0.30 mg. of protein) was added, and after incubation at 37° for the given time intervals, 0.5-ml. aliquots were withdrawn, treated with 0.2 ml. of 10 per cent trichloroacetic acid, and centrifuged. A 0.2-ml. aliquot of the supernatant solution was used for the determination of KDHP (8).

† Same as above, except that substrates (1 μmole of each) were preincubated in a volume of 1.94 ml. and 0.02 ml. (0.2 mg. of protein) of muscle aldolase was added with the acetone fraction. The SDP used in this experiment was pure by enzymatic assay (11). In a control experiment without muscle aldolase there was no KDHP formation.

SUMMARY

The conversion of sedoheptulose 1,7-diphosphate to 5-dehydroquinic acid in *Escherichia coli* extracts was shown to be due to (*a*) the cleavage of sedoheptulose 1,7-diphosphate to D-erythrose 4-phosphate and dihydroxyacetone phosphate; (*b*) the oxidation of dihydroxyacetone phosphate to phosphoenolpyruvate; and (*c*) the synthesis of 5-dehydroquinic acid from erythrose 4-phosphate and phosphoenolpyruvate.

REFERENCES

1. Kalan, E. B., Davis, B. D., Srinivasan, P. R., and Sprinson, D. B., *J. Biol. Chem.*, **223**, 907 (1956).
2. Srinivasan, P. R., Sprinson, D. B., Kalan, E. B., and Davis, B. D., *J. Biol. Chem.*, **223**, 913 (1956).
3. Srinivasan, P. R., Shigeura, H. T., Sprecher, M., Sprinson, D. B., and Davis, B. D., *J. Biol. Chem.*, **220**, 477 (1956).
4. Srinivasan, P. R., Katagiri, M., and Sprinson, D. B., *J. Am. Chem. Soc.*, **77**, 4943 (1955).
5. Davis, B. D., *J. Biol. Chem.*, **191**, 315 (1951).
6. Davis, B. D., and Weiss, U., *Arch. exptl. Pathol. Pharmakol.*, **220**, 1 (1953).
7. Rothschild, J., *Federation Proc.*, **16**, 239 (1957).
8. Srinivasan, P. R., and Sprinson, D. B., *J. Biol. Chem.*, **234**, 716 (1959).
9. Ballou, C. E., Fischer, H. O. L., and MacDonald, D. L., *J. Am. Chem. Soc.*, **77**, 5967 (1955).
10. Horecker, B. L., Smyrniotis, P. Z., Hiatt, H. H., and Marks, P. A., *J. Biol. Chem.*, **212**, 827 (1955).
11. Racker, E., and Schroeder, E. A. R., *Arch. Biochem. Biophys.*, **74**, 326 (1958).
12. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941–1942).
13. Horecker, B. L., Smyrniotis, P. Z., and Klenow, H., *J. Biol. Chem.*, **205**, 661 (1953).
14. Krebs, H. A., *Bull. Johns Hopkins Hosp.*, **95**, 45 (1954).
15. Mitsuhashi, S., and Davis, B. D., *Biochim. et Biophys. Acta*, **15**, 268 (1954).
16. Srinivasan, P. R., and Sprinson, D. B., *Federation Proc.*, **15**, 360 (1956); **16**, 253 (1957).

2-Keto-3-deoxy-D-*arabo*-heptonic Acid 7-Phosphate Synthetase*

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In the preceding paper (1) it was demonstrated that cell-free extracts of *Escherichia coli* converted D-erythrose 4-phosphate and phosphoenolpyruvate almost quantitatively to 5-dehydroquinic acid. It was postulated that the initial reaction in this conversion is a condensation of phosphoenolpyruvate and D-erythrose 4-phosphate to yield inorganic phosphate and 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate. The present paper describes the purification and properties of the enzyme carrying out this reaction, and the identification of the product as KDHP[1] by comparison with the chemically synthesized compound. The name KDHP synthetase is suggested for this enzyme. The later stages of the enzyme purification were greatly aided by the discovery of Weissbach and Hurwitz (3) (communicated to us before publication) that β-formylpyruvic acid, derived from KDHP by the action of periodate, reacts with thiobarbituric acid to give an intense pink color with an absorption maximum at 549 mμ. A part of these results was published in preliminary form (4).

EXPERIMENTAL

Materials—The dimethylacetal of cyclohexylammonium erythrose-4-P was prepared according to the method of Ballou *et al.* (5), and used as sodium erythrose-4-P (5). Barium P-enolpyruvate was a generous gift of Mr. W. E. Pricer, Jr., and was converted to the potassium salt. KDHP and 3-deoxy-D-*arabo*-heptonic acid 7-phosphate (configuration of α-hydroxyl unknown) were prepared according to an unpublished procedure.[2] 2-Keto-3-deoxygluconic acid 6-phosphate and 2-keto-3-deoxygalactonic acid were gifts of Professor M. Doudoroff. Sedoheptulose 1,7-diphosphate, pure by enzymatic assay (6), was a gift of Professor E. Racker. Sedoheptulose 7-phosphate was a gift of Professor B. L. Horecker. D-Glucosamine 6-phosphate and *N*-acetyl-D-glucosamine 6-phosphate were gifts of Professor S. Roseman. D-Erythrose (from the diacetamide) and 2,3-diketovaleric acid were gifts of Professor Z. Dische. The cyclohexylammonium salts of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate were gifts of Professor C. E. Ballou.

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† Career Investigator of the American Heart Association.

[1] The following abbreviations are used: erythrose-4-P, D-erythrose 4-phosphate; KDHP, 2-keto-3-D-*arabo*-heptonic acid 7-phosphate; EDTA, sodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

KDHP is correctly named as abover ather than, as in previous publication, 2-keto-3-deoxy-7-phospho*gluco*heptonic acid.

[2] D. B. Sprinson and J. Rothschild, in preparation (*cf.* (2)).

The mutant strains used in this work were kindly furnished by Professor B. D. Davis. Yeast hexokinase (30 per cent pure) was a gift of Professor E. Racker. Neurospora DPNase (60 per cent acetone fraction) was a gift of Professor N. O. Kaplan.

The following materials were obtained from commercial sources: 3-phosphoglyceric acid, fructose 1,6-diphosphate, and ribose 5-phosphate, Schwarz Laboratories, Inc.; 2-thiobarbituric acid, Eastman-Kodak; crystalline bovine serum albumin, Armour Laboratories; diethylaminoethyl cellulose, Brown and Company, Berlin, N. H.; protamine sulfate, Nutritional Biochemicals Corporation; β-ketobutyraldehyde dimethylacetal (a gift), Henley and Company, Inc., New York; DPN, Pabst Laboratories; ATP, ADP, and DPNH, Sigma Chemical Company; crystalline lactic dehydrogenase (containing P-enolpyruvate-ATP transphosphorylase), Worthington Biochemical Corporation.

Analytical Procedures—Protein was determined by the method of Lowry *et al.* (7); however, before the addition of protamine it was also estimated spectrophotometrically (8).[3] Inorganic phosphate was determined by the method of Fiske and SubbaRow (9). P-enolpyruvate was measured enzymatically according to Kornberg and Pricer (10). Light absorption measurements were carried out on a Cary Recording Spectrophotometer or a Beckman model B Spectrophotomer.

Enzyme Purification—E. coli mutant 83-24 (5) was grown for 18 hours with aeration at 37° in medium A (11) supplemented with 0.2 per cent yeast extract (Difco) and 0.2 per cent casein hydrolysate. The cells were harvested by centrifugation at 2°, washed with cold water, suspended in M/30 phosphate buffer, pH 7.4 (20 ml./5.0 gm. of wet bacteria), and disrupted by means of sonic oscillation for 30 minutes in a 9-kc. Raytheon oscillator cooled with circulating ice water. Centrifugation at 13,000 × *g* in a Spinco preparative centrifuge yielded a clear greenish yellow solution containing 16 mg. of protein per ml. All subsequent operations were carried out at 2°.

Two hundred ml. of the cell free extract was treated with 28 ml. of 2 per cent protamine sulfate solution,[3] and the precipitate was removed by centrifugation. To 218 ml. of the supernatant solution were added slowly with stirring 54 gm. of $(NH_4)_2SO_4$ and, after stirring for another 20 minutes, the precipitate was removed by centrifugation and discarded. The procedure was repeated with 21 gm. of $(NH_4)_2SO_4$ on the supernatant solution. The precipitate was removed by centrifugation, dissolved in 45 ml. of M/30 potassium phosphate buffer pH 7.4 and dialyzed against the same buffer (4 changes of 2 liters each at 2-hour intervals).

[3] 0.5 ml. of 2 per cent protamine solution for every 70 mg. of protein as estimated spectrophotometrically (8).

The rest of the fractionation procedure was best carried out on a smaller scale. Twenty ml. of the dialysate was adjusted to pH 5.4 with 1 N acetic acid and the precipitate was removed by centrifugation. The supernatant solution (cooled by an ice bath) was treated with 17 ml. of acetone (precooled to −15°) slowly with stirring and the precipitate was removed by centrifugation and discarded. The supernatant solution was treated similarly with 12 ml. of acetone, and the precipitate was removed by centrifugation, dissolved in 5 ml. of M/30 potassium phosphate buffer, pH 7.4, and dialyzed against 2 liters of 0.01 M phosphate buffer, pH 6.8, as described previously.

The diethylaminoethyl cellulose column used in the next step was prepared as follows. To 3 gm. of diethylaminoethyl cellulose suspended in 100 ml. of H_2O at room temperature, 1.5 ml. of 1.0 M KH_2PO_4 was added with stirring. (The pH of the solution was 6.8.) The cellulose was removed by filtration on a Buchner funnel, washed several times with 0.01 M potassium phosphate buffer, pH 6.8, and suspended in 70 ml. of the same buffer. Ten ml. of this suspension was placed on a chromatographic column (13 mm. diameter) and packed by mild suction (length, 35 mm.). The column was transferred to a cold room at 2° and equilibrated with cold buffer by allowing approximately 50 ml. to pass through the column (2 hours).

One ml. of the dialyzed acetone fraction was placed on the column and eluted successively with 5.0 ml. each of phosphate buffer, pH 6.8, of the following molar concentrations (flow rate 5 ml. in 20 minutes): 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.10, 0.16, and 0.20. The desired enzyme activity was present in the two 0.1 M fractions. Four such fractions (from two column operations) were combined (3.8 mg. of protein), and immediately concentrated by precipitation with $(NH_4)_2SO_4$ (80 per cent saturation). The precipitate was dissolved in 0.8 ml. M/30 potassium phosphate buffer pH 7.4, dialyzed against the same buffer, and clarified by centrifugation. Recovery of protein was 1.8 mg.

A summary of the enzyme purification is given in Table I. The $(NH_4)_2SO_4$ and acetone fractions were stable for at least several weeks at −15°. The cellulose fractions were stored at 2°.

Assay—The activity of the enzyme was determined by measuring the amount of KDHP formed from erythrose-4-P and P-enolpyruvate. A unit of enzyme was defined as that amount of enzyme which will form 0.1 μmole of KDHP in 5 minutes under the following conditions.

The incubation mixture contained 100 μmoles of potassium phosphate buffer (pH 6.4), 0.5 μmole each of erythrose-4-P and P-enolpyruvate, and enzyme fraction in a total volume of 1.0 ml. The reaction was started by the addition of enzyme after preincubation of the other constituents at 37° for 10 minutes. After 5 minutes at 37° the reaction was stopped by the addition of 0.4 ml. of 10 per cent trichloroacetic acid and the mixture was centrifuged. Aliquots were removed for the estimation of KDHP by cleavage with periodic acid to β-formylpyruvic acid (butyric acid-2,4-dione), and estimation of the latter by the color produced with thiobarbituric acid.

Estimation of KDHP—0.01 to 0.05 μmoles of compound in 0.25 ml. of solution was treated with 0.25 ml. of 0.025 M periodic acid in 0.125 N H_2SO_4. After 45 minutes at room temperature, 0.5 ml. of 2 per cent sodium arsenite in 0.5 N HCl was added to destroy the excess periodate (2 minutes at room temperature). Two ml. of 0.3 per cent thiobarbituric acid solution were added and the tubes were placed in a boiling water bath for 5 minutes.

TABLE I

Purification of KDHP synthetase

Fraction	Volume	Protein concentration	Total activity	Specific activity
	ml.	*mg./ml.*	*units*	*units/mg.*
Crude extract	200	16	1080	0.34
Ammonium sulfate (40–55%)	45	15	790	1.20
Acetone (47–59%)	11	7.3	440	5.6
Diethylaminoethyl cellulose (0.1 M phosphate eluates)	110	0.19	420	20

(To prepare the thiobarbituric acid solution, 300 mg. of thiobarbituric acid were dissolved in about 70 ml. of H_2O with the aid of 3 ml. of 1 N NaOH; 2.5 ml. of 1 N HCl was added, and the pH adjusted to 2 in a final volume of 100 ml.) After cooling in a water bath at 40° the pink color was measured immediately at 549 mμ in a spectrophotometer against a blank run with water under the same conditions. The rate of production of chromogenic material from KDHP by periodic acid is shown in Fig. 1.

Paper-chromatographic Identification—A solution equivalent to 3 to 5 μg. of KDHP was spotted on acid-washed Whatman No. 1 paper and developed for 24 hours by descending chromatography with a mixture of tertiary amyl alcohol-formic acid (98 per cent)-water (3:3:1). The paper was dried at room temperature and sprayed with 0.1 M periodic acid in 0.125 N H_2SO_4. After 20 minutes at room temperature the paper was sprayed with 10 per cent sodium arsenite in 0.5 N HCl. The paper became colored by liberated iodine which was removed by further reaction with

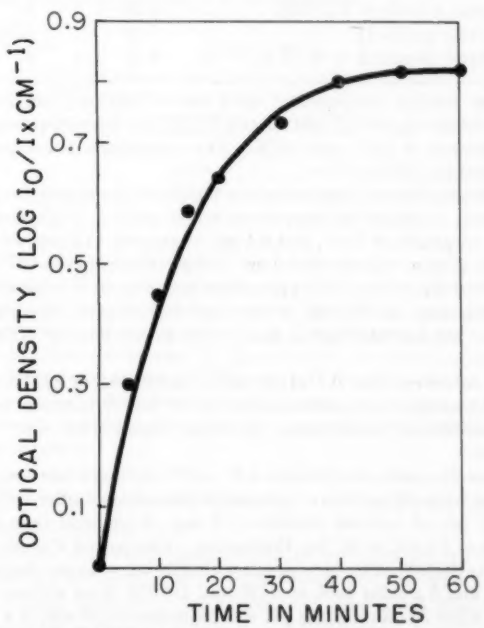


FIG. 1. Development of color with KDHP in the periodate-thiobarbiturate reaction. The reaction time with periodate was varied as indicated, 0.04 μmoles of KDHP being treated as described under "Methods."

TABLE II

Behavior of various compounds in periodate-thiobarbiturate reaction

Compound	λ_{max}	ϵ_{max}
	mμ	
KDHP*	549	7.2×10^4
2-Keto-3-deoxygalactonic acid*	549	
2-Keto-3-deoxygluconic acid 6-P*	549	
1,3,4,5,6-Pentahydroxycyclohexanecarboxylic acid*†	549	
Malondialdehyde (1,1,3,3-tetramethoxypropane)‡	532	13.0×10^4
3-Deoxy-D-*arabo*-heptonic acid 7-P*	(none)	
β-Ketobutyraldehyde dimethyacetal‡	542	3000
Glyoxal‡	546	2560
Glycolaldehyde‡	517	136
Acetoacetic acid, acetopyruvic acid (2,4-diketovaleric), dimedon, acetonylacetone‡	(none)	

* Treated with periodate and thiobarbiturate.

† Shikimic acid was hydroxylated with OsO_4 to give the pentahydroxy acid and then treated with periodate to give β-formylpyruvic acid (13).

‡ Treated with thiobarbiturate only.

TABLE III

*Conversion of P-enolpyruvate–erythrose-4-P condensation product and of synthetic KDHP to 5-dehydroquinate**

Substrate†	Amount incubated	Dehydroquinate formed
	μmoles	μmoles
Synthetic KDHP	0.20	0.17
Synthetic KDHP + EDTA‡	0.20	0
Enzymatic product§	0.27	0.24
Enzymatic product§ + EDTA‡	0.27	0

* The enzyme preparation[4] used was a fraction from *E. coli* strain 83-24 capable of converting KDHP to dehydroquinate in the presence of Co^{++} and DPN. This conversion is completely inhibited by EDTA.

† The reaction mixture contained KDHP or enzymatic product, 50 μmoles of potassium phosphate buffer pH 7.4, 0.25 μmole of DPN, 1.0 μmole of Co^{++}, and 0.1 ml. of enzyme (1.5 mg. of protein) in a total volume of 1.0 ml. After incubation at 37° for 1 hour, 0.02 ml. of 6 N HCl was added, proteins were removed by centrifugation, and 0.2 ml. of the clear supernatant solution was used for the microbiological assay with *E. coli* mutant A170-143 S_1 (16).

‡ 0.1 ml. of enzyme, 0.1 ml. of buffer, and 0.1 ml. of EDTA solution (0.4 μmole) were preincubated at 37° for 10 minutes before the addition of substrates. In these experiments Co^{++} was omitted.

§ 3 μmoles each of erythrose-4-P and P-enolpyruvate were incubated with 300 μmoles of potassium phosphate buffer, pH 7.4 and 0.3 ml. of acetone fraction (1.3 mg. of protein) in a total volume of 3.0 ml. at 37° for 15 minutes. Unchanged P-enolpyruvate was destroyed by incubation with 0.04 ml. of lactic dehydrogenase and 5 μmoles each of ADP and DPNH (final volume 3.44 ml.). After 30 minutes the pH was adjusted to 2.0 with 6 N HCl and then brought to 6.0 with 4 N NaOH. The precipitated proteins were removed by centrifugation. Aliquots of the supernatant solution were then used for the determination of KDHP and for conversion to dehydroquinate.

arsenite. This was repeated until further application of arsenite did not produce the color of iodine. (Two to three sprayings were necessary.) The paper was allowed to dry at room temperature, sprayed with 0.6 per cent thiobarbituric acid (pH 2.0), and dried in an oven at 90° for 5 minutes. KDHP and 2-keto-3-deoxygalactonic acid appeared as pink spots. The R_F values for these two compounds were 0.49 and 0.55, respectively.

A similar procedure was used with a mixture of ethanol-water-acetic acid (75:24:1) on Whatman No. 50. The R_F of KDHP was 0.26.

RESULTS

Scope and Sensitivity of Periodate-Thiobarbiturate Reaction

Several compounds structurally related to β-formylpyruvic acid, or expected to give rise to it on periodate oxidation, were tested in the procedure described under "Estimation of KDHP." The results are summarized in Table II. Simple aliphatic aldehydes, α,β-diketones, β-keto acids, and β-formyllactic acid (derived from 3-deoxy-D-*arabo*-heptonic acid 7-phosphate[2]) did not yield colored products under these conditions. Glycolaldehyde and glyoxal gave brown solutions with very small absorptions at 517 and 546 mμ, respectively. In addition to the first four compounds in Table II, which would be expected to yield β-formylpyruvic acid with periodate, only β-keto-butyraldehyde dimethylacetal gave a pink color, but it was quite unstable and weak.

With KDHP, the optimal reaction time with periodate was 45 minutes (Fig. 1). However, this could be reduced to 20 minutes by the addition of 10^{-2} molar concentrations of glycine or glycylglycine. The function of these compounds is unknown, and is under further investigation.

Identification of Product of Erythrose-4-P and P-enolpyruvate Condensation as KDHP

Chromatographic and Chemical Comparison with Synthetic KDHP—Erythrose-4-P and P-enolpyruvate were incubated with enzyme and aliquots were spotted on acid-washed Whatman No. 1 filter paper along with synthetic KDHP, erythrose-4-P, and P-enolpyruvate. The papers were developed with tertiary amyl alcohol-formic acid-water. KDHP was detected by the periodate-thiobarbituric acid reaction as indicated earlier, and also by spraying with semicarbazide (14, 15) (0.1 per cent semicarbazide hydrochloride in 0.15 per cent sodium acetate). The semicarbazide-treated paper was dried in an oven at 95° for 10 minutes. Under ultraviolet irradiation the semicarbazones were revealed as dark spots. By either method, a strong spot corresponding to KDHP (R_F = 0.49) was present in the enzymatic reaction mixture. Identical R_F values of 0.26 were also found for synthetic KDHP and enzymatic product with ethanol-water-acetic acid on Whatman No. 50 paper.

Biological Activity of Enzymatic and Synthetic Product—Synthetic KDHP and the product of the P-enolpyruvate–erythrose-4-P condensation were incubated with an enzyme preparation[4] capable of converting KDHP to 5-dehydroquinate, and the latter was estimated microbiologically (16). The results, presented in Table III, show identical behavior for the enzymatically formed product and for synthetic KDHP.

[4] P. R. Srinivasan and D. B. Sprinson, unpublished results (*cf.* (12)).

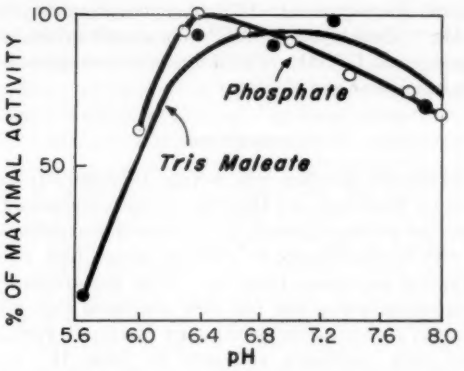


FIG. 2. The pH optimum of KDHP synthetase. For the phosphate curve the incubation procedure was exactly as described under "Assay", with 0.01 ml. of the acetone fraction (51 μg. of protein). The Tris-maleate reaction mixtures were similarly prepared with 0.5 ml. of 0.2 M buffer in a total volume of 1.0 ml. The pH of the reaction mixtures was determined on duplicate solutions prepared in the same way.

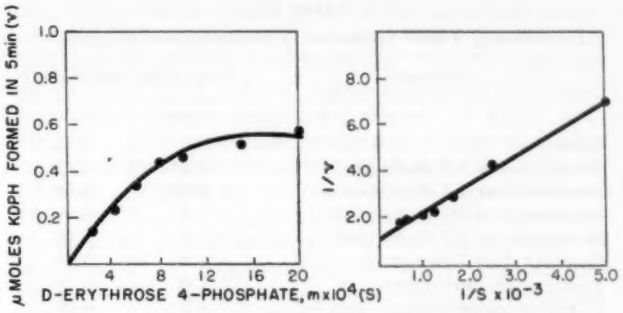


FIG. 3. Rate dependence of P-enolpyruvate–erythrose-4-P condensation on the erythrose-4-P concentration. In addition to erythrose-4-P, the reaction mixture contained 1.0 μmole of P-enolpyruvate, 50 μmoles of potassium phosphate buffer (pH 6.4), and 0.01 ml. of acetone fraction (51 μg. of protein) in a final volume of 0.5 ml. See "Assay" for procedure.

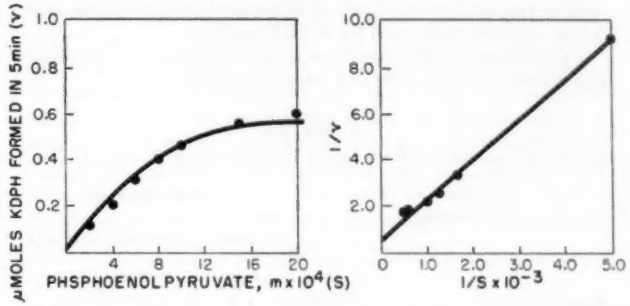


FIG. 4. Rate dependence of the P-enolpyruvate–erythrose-4-P condensation on the P-enolpyruvate concentration. The procedure was the same as in Fig. 3 using 1.0 μmole of erythrose-4-P and varying amounts of P-enolpyruvate.

Properties of KDHP Synthetase

pH Optimum of KDHP Formation—The effect of pH on the rate of formation of KDHP from P-enolpyruvate and erythrose-4-P is shown in Fig. 2. The pH optimum is at 6.4 in potassium phosphate buffer. With Tris maleate-buffer, the H^+ ion concentration had little effect on the reaction between pH 6.4 and 7.4.

Specificity—Erythrose-4-P cannot be replaced by D-erythrose, D-glyceraldehyde 3-phosphate, ribose 5-phosphate, glucose 6-phosphate, glucosamine 6-phosphate and *N*-acetylglucosamine 6-phosphate. Pyruvate or pyruvate plus ATP cannot substitute for P-enolpyruvate. It would have been of interest to test the isomers of erythrose-4-P, but these were not available.

Effect of Substrate Concentration—The effect on the reaction rate of varying concentrations of erythrose-4-P (Fig. 3) and P-enolpyruvate (Fig. 4) was determined. These results have been plotted according to Lineweaver and Burk (17) for the determination of K_m values. The K_m for erythrose-4-P was found to be 1.2×10^{-3} M and for P-enolpyruvate 3.5×10^{-3} M.

Stoichiometry—The disappearance of P-enolpyruvate and the formation of KDHP and inorganic phosphate were measured, and are recorded in Table IV. The stoichiometry agrees with the expected bimolecular condensation of erythrose-4-P and P-enolpyruvate.

Inhibitors—With the acetone fraction, EDTA (4×10^{-4} M) did not inhibit the reaction.[5] Co^{++}, Zn^{++}, Mg^{++} at a concentration of 2×10^{-3} M had no effect on the condensation. Fluoride, sodium arsenite, azide, and iodoacetate did not affect the rate of formation of KDHP. *p*-Chloromercuribenzoate (2×10^{-5} M) inhibited the reaction completely and this inhibition could be reversed by cysteine.

Another inhibition, discussed in the previous paper (1), is exhibited by sedoheptulose 1,7-diphosphate and several other phosphorylated carbohydrates (Table V). The most inhibitory of these is the α-hydroxy analogue of KDHP which is an intermediate in its chemical synthesis.[2] However, in another experiment, KDHP was not inhibitory, at initial concentrations which were 60 per cent of that of the erythrose-4-P.

TABLE IV

*Stoichiometry of KDHP synthetase reaction**

Experiment No.	P-enolpyruvate disappeared	Orthophosphate formed	KDHP formed
	μmoles	*μmoles*	*μmoles*
1	1.53	1.62	1.56
2		1.68	1.68

* The reaction mixture (3 ml.) contained 3 μmoles each of erythrose-4-P and P-enolpyruvate, 240 μmoles of Tris-maleate buffer, pH 7.0, and 0.25 ml. of acetone fraction (1.1 mg. of protein) which was dialyzed against 0.2 M Tris-maleate buffer, pH 7.0. The reaction was started by the addition of enzyme after preincubation of the other constituents at 37° for 10 minutes. Before and after an incubation period of 15 minutes aliquots were withdrawn for the determination of P-enolpyruvate, KDHP, and orthophosphate.

[5] In crude extracts and in the $(NH_4)_2SO_4$ fractions KDHP is utilized in the next reaction of aromatic biosynthesis, *i.e.* its conversion to dehydroquinate. This conversion (which proceeds at a considerably slower rate than the KDHP synthetase reaction) requires DPN and Co^{++} (12). The addition of either DPNase or EDTA, although inhibiting the conversion of KDHP to dehydroquinate (12), does not inhibit KDHP formation in the acetone and cellulose fractions. Nevertheless, the addition of DPNase results in an 81 and 50 per cent inhibition of KDHP formation in the crude and $(NH_4)_2SO_4$ fractions, respectively, while the addition of EDTA results in a 97 and 44 per cent inhibition. These unexpected inhibitions are being further investigated.

TABLE V

*Inhibition of KDHP synthetase by phosphorylated compounds**

Compound†	Amount added	Yield of KDHP
	μmoles	*μmoles*
None	0	0.14
Sedoheptulose 1,7-diphosphate	0.25	0.12
Sedoheptulose 1,7-diphosphate	0.50	0.08
Sedoheptulose 1,7-diphosphate	1.0	0.06
Sedoheptulose 1,7-diphosphate	1.5	0.06
Sedoheptulose 7-phosphate	1.0	0.10
Fructose 1,6-diphosphate	1.0	0.10
3-Deoxy-D-*arabo*-heptonic acid 7-P	1.0	0.03

* 0.25 μmole of erythrose-4-P, 0.30 μmole of P-enolpyruvate, 50 μmoles of potassium phosphate buffer pH 7.0, and inhibitor were preincubated at 37° for 10 minutes. After the addition of 0.01 ml. of acetone fraction (73 μg. of protein) the reaction mixture (0.5 ml.) was incubated at 37° for 15 minutes, and deproteinized by the addition of 0.2 ml. of 10 per cent trichloroacetic acid. 0.2 ml. aliquots were used for KDHP analysis.

† Dihydroxyacetone phosphate, D-glyceraldehyde 3-phosphate, and 3-phospho-D-glyceric acid were not inhibitory.

TABLE VI

*Extent of reaction in P-enolpyruvate–erythrose-4-P condensation**

Additions	KDHP formed at		
	15 minutes	30 minutes	60 minutes
	μmoles	*μmoles*	*μmoles*
None	1.2	1.2	1.2
Crystalline bovine serum albumin	1.2	1.2	1.2
2-Mercaptoethanol†	1.6	1.8	1.9

* The incubation mixture (2 ml.) contained 2.0 μmoles of erythrose-4-P, 2.5 μmoles of P-enolpyruvate, 200 μmoles of potassium phosphate buffer, pH 6.4, 0.08 ml. of acetone fraction (0.6 mg. of protein), and, when added, 4.0 mg. of serum albumin, or 4 μmoles of 2-mercaptoethanol. The latter was preincubated with the enzyme for 10 minutes at 37° before addition of the other components.

† At this concentration of 2-mercaptoethanol (2×10^{-3} M) the thiobarbiturate assay for KDHP was inhibited 27 to 30 per cent. The values reported in the table were corrected accordingly.

Extent of Reaction—In crude extracts erythrose-4-P and P-enolpyruvate are nearly quantitatively converted to 5-dehydroquinate (approximately 90 per cent). With purified preparations the condensation proceeds very rapidly in the first 10 minutes and stops when the yield of KDHP is approximately 60 per cent. That this inactivation is not simply due to surface denaturation of dilute protein solutions, but to oxidation of sulfhydryl groups is shown by the results in Table VI. Serum albumin had no protective effect, whereas in the presence of 2-mercaptoethanol the reaction went essentially to completion.[6]

Reversibility—All attempts to reverse the condensation reaction were unsuccessful. Thus KDHP disappearance could not be detected when incubated with enzyme in the presence of inorganic phosphate under the following conditions: (*a*) with ADP, Mg^{++}, and P-enolpyruvate-ATP transphosphorylase; (*b*) with ADP, Mg^{++}, P-enolpyruvate-ATP transphosphorylase, lactic dehydrogenase and DPNH; (*c*) with the substrates and enzymes of (*b*) plus hexokinase and glucose.

[6] A yield of 90 to 95 per cent KDHP may be considered quantitative, since in the preparation of erythrose-4-P solutions from cyclohexylammonium erythrose-4-P dimethylacetal the yield of erythrose-4-P is approximately 90 per cent (5).

DISCUSSION

Thiobarbiturate Reaction with β-Keto Aldehydes—It was first observed by Weissbach and Hurwitz (3) that a compound resulting from the action of periodate on 2-keto-3-deoxyaldonic acids reacts with thiobarbiturate to yield an intense pink color with an absorption maximum at 549 mμ. From the structure of several compounds giving this test they concluded that β-formylpyruvic acid is responsible for the color reaction. Further support for their conclusion is shown in Table II. Although β-formylpyruvic acid has not been obtained in pure form, it has previously been isolated as its bis-2,4-dinitrophenylhydrazone from 1,3,4,5,6-pentahydroxycyclohexanecarboxylic acid (13). When the periodate-thiobarbiturate reaction was applied to this compound, an absorption curve identical with that given by KDHP and related compounds was obtained. Since β-ketobutyraldehyde, unlike several other dicarbonyl compounds, also gave a pink color[7] with thiobarbiturate (λ_{max} = 542 mμ), it would appear that the structure of $R \cdot CO \cdot CH_2 \cdot CHO$ is essential for color formation. The previous application of this test to malondialdehyde (λ_{max} = 532 mμ (21–23)) would exemplify the case where R = H. Since β-formylpyruvic acid reacts slowly with periodate (3),[8] the molar absorbancy index of KDHP recorded in Table II may represent a minimal value.

Properties of KDHP Synthetase—It may be seen from Table I that the fraction obtained from the diethylaminoethyl cellulose column represented a 60-fold purification of the KDHP synthetase. In a preliminary investigation this fraction appeared to be homogeneous in the Spinco analytical centrifuge.[9] Most of the work reported in this paper was done with the acetone fraction, which was shown to be free of aldolase (by its inability to cleave sedoheptulose 1,7-diphosphate), P-enolpyruvate-ATP transphosphorylase, the enzyme system for the conversion of KDHP to 5-dehydroquinate[4], dehydroquinic-dehydrase (26), and DPNH oxidase.

As far as is known at present only erythrose-4-P and P-enolpyruvate are condensed by this enzyme. That the product of the condensation is KDHP is shown by comparison of the enzymatic product with synthetic KDHP in chemical and chromatographic behavior, and in enzymatic conversion to dehydroquinate. The enzyme preparation used in the latter test is inactive with 3-deoxy-D-*arabo*-heptonic acid 7-phosphate, 2-keto-3-deoxy-D-*arabo*-heptonic acid (dephosphorylated KDHP), 2-keto-3-deoxygluconic acid 6 phosphate, or any related compounds. Furthermore, although erythrose-4-P disappearance

[7] β-Ketobutyraldehyde would not be expected to show maximal color development under the conditions of the test, since it condenses in acid solution to 1,3,5-triacetyl benzene (18–20). The fading of the color produced by β-ketobutyraldehyde may be due to a reversal of the reaction with thiobarbiturate favored by the aromatization of the aldehyde.

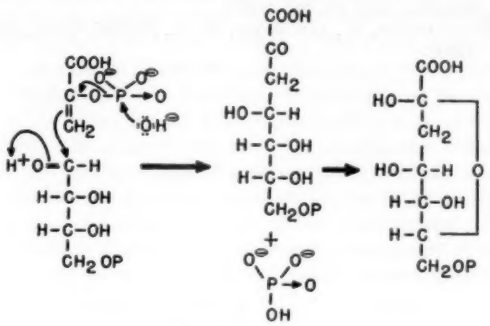
[8] Presumably this decomposition (3) consists of hydroxylation of the active methylene carbon (24, 25) to yield β-formylhydroxypyruvic acid, followed by periodate cleavage to formate and mesoxalic semialdehyde, decarboxylation to glyoxal, and periodate cleavage to 2 molecules of formate.

[9] We are indebted to Dr. H. C. Lawler for aid with this determination.

was not measured, the stoichiometry of the reaction (Table IV) is in accord with the concept that erythrose-4-P and P-enolpyruvate are condensed to form KDHP and inorganic phosphate. The specificity of the enzyme for its substrates suggests its use for the assay of either erythrose-4-P or P-enolpyruvate.

KDHP synthetase is apparently a sulfhydryl enzyme, since its inhibition by *p*-chloromercuribenzoate is reversed by cysteine. Although it is stable during preparation, the purer fractions (starting with the acetone fraction) showed a sharp halt in activity after 10 to 15 minutes, when the yield of KDHP was 50 to 60 per cent of the theoretical. However, when 2-mercaptoethanol was added, complete conversion of the substrates to KDHP occurred. This behavior may be due to a greater tendency of the enzyme to oxidation in the presence of its substrates.

Mechanism of Condensation Reaction—The condensation of erythrose-4-P and P-enolpyruvate may be tentatively regarded (as shown in Scheme I) as a nucleophilic attack on P-enolpyruvate,



SCHEME I

by a reagent here symbolized as OH^-, resulting in the release of phosphate and the formation of the open chain form of KDHP. It is analogous to the CO_2 fixation reaction (27, 28): P-enolpyruvate$^{=}$ + HCO_3^- → oxaloacetate$^{=}$ + $HPO_4^{=}$. Since the pyranose form of KDHP is highly favored both in the solid state and in solution,[2] cyclization to this form would presumably occur spontaneously. The effect on KDHP formation of 6- and 7-carbon phosphorylated carbohydrates (Table V) could be due to an inhibition of the condensation reaction, suggested above, or of the release of KDHP from the enzyme surface.

Recent studies on the mechanism of action of yeast and muscle aldolase have demonstrated the formation of a dihydroxyacetone phosphate-aldolase complex capable of exchanging a carbon-bound hydrogen atom with water (29, 30) and showing strong light absorption between 250 and 233 mμ (31). A carbanion ↔ enediol structure of dihydroxyacetone phosphate was postulated to undergo the exchange reaction, as well as the condensation with aldehydes. In the KDHP synthetase reaction, however, an analogous pyruvate carbanion could not be an intermediate, since its protonation would create an insurmountable energy barrier to the reformation of P-enolpyruvate. The concerted mechanism is, therefore, more reasonable. For this reason the name originally given (4) to the enzyme described here is changed from "P-enolpyruvate-erythrose-4-P aldolase" to KDHP synthetase. Further work is now in progress to elucidate the mechanism of the P-enolpyruvate-erythrose-4-P condensation.

On the assumption that the free energy change in the formation of the C-3 to C-4 linkage of KDHP is small,[10] the free energy change of the KDHP synthetase reaction, P-enolpyruvate$^{=}$ + erythrose-4-P$^{=}$ + H_2O → $HPO_4^{=}$ + KDHP$^{=}$, may be considered to be approximately the same as that of the hydrolysis of P-enolpyruvate (33): P-enolpyruvate$^{=}$ + H_2O → pyruvate$^-$ + $HPO_4^{=}$; ΔF^0 = 13.6 kilocalories. The apparent irreversibility of the KDHP synthetase reaction may be due not only to the large negative value of ΔF^0 for the conversion of P-enolpyruvate to "pyruvate" and orthophosphate, but also to the stability of the pyranose form. In the cleavage of fructose 1-phosphate derivatives by aldolase (34) the pyranoside fructose 1-phosphate can be calculated to be more stable by 3.6 kilocalories than the open chain 5,6-dideoxyfructose 1-phosphate. The participation of P-enolpyruvate rather than of pyruvate in the condensation with erythrose-4-P may be of advantage in a biosynthetic reaction requiring KDHP. since 2-keto-3-deoxyaldonic acids, *e.g.* 2-keto-3-deoxygluconic acid 6-P (15, 35) and *N*-acetylneuraminic acid (31), are readily degraded to pyruvate and the corresponding aldehydes (glyceraldehyde 3-phosphate, and *N*-acetyl-D-mannosamine, respectively).

SUMMARY

An enzyme which catalyzes the condensation of D-erythrose 4-phosphate and phosphoenolpyruvate to form 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate and inorganic phosphate was purified from extracts of *Escherichia coli*. After a 60-fold purification it appeared homogeneous in the ultracentrifuge. The name 2-keto-3-deoxy-D-*arabo*-heptonic acid 7 phosphate synthetase is suggested for this enzyme.

The yields of 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate were quantitative, and attempts to reverse the reaction were unsuccessful. Only phosphoenolpyruvate and D-erythrose 4-phosphate were activated by the enzyme. It had the properties of a sulfhydryl protein and showed maximal activity in the presence of 2-mercaptoethanol. No cofactor requirements could be demonstrated. The reaction was inhibited by fructose 1,6-diphosphate, sedoheptulose 1,7-diphosphate, and 3-deoxy-D-*arabo*-heptonic acid 7-phosphate.

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REFERENCES

1. SRINIVASAN, P. R., KATAGIRI, M., AND SPRINSON, D. B., *J. Biol. Chem.*, **234**, 713 (1959).
2. ROTHSCHILD, J., *Federation Proc.*, **16**, 239 (1957).
3. WEISSBACH, A., AND HURWITZ, J., *J. Biol. Chem.*, **234**, 705 (1959).
4. SRINIVASAN, P. R., AND SPRINSON, D. B., *Federation Proc.*, **17**, 315 (1958).
5. BALLOU, C. E., FISCHER, H. O. L., AND MACDONALD, D. L., *J. Am. Chem. Soc.*, **77**, 5967 (1955).
6. RACKER, E., AND SCHROEDER, E. A. R., *Arch. Biochem. Biophys.*, **74**, 326 (1958).
7. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
8. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **310**, 384 (1941–1942).
9. FISKE, C. H., AND SUBBAROW, Y., *J. Biol. Chem.*, **66**, 375 (1925).

[10] A ΔF^0 value of approximately −1000 calories can be calculated from the published data (32) for the reaction: pyruvate$^-$ + *N*-acetyl-D-mannosamine → *N*-acetylneuraminate$^-$.

10. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193**, 481 (1951).
11. Davis, B. D., and Mingioli, E, S,, *J. Bacterial.*, **60**, 17 (1950).
12. Srinivasan, P. R., and Sprinson, D. B., *Federation Proc.*, **15**, 360 (1956); **16**, 253 (1957).
13. Srinivasan, P. R., Shigeura, H. T., Sprecher, M., Sprinson, D. B., and Davis, B. D., *J. Biol. Chem.*, **220**, 477 (1956).
14. Magasanik, B., and Umbarger, H. E., *J. Am. Chem. Soc.*, **72**, 2308 (1950); Umbarger, H. E., and Magasanik, B., *J. Am. Chem. Soc.*, **74**, 4253 (1952).
15. MacGee, J., and Doudoroff, M., *J. Biol. Chem.*, **210**, 617 (1954).
16. Davis, B. D., and Weiss, U., *Arch. exptl. Pathol. Pharmakol.*, **220**, 1 (1953).
17. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
18. Claisen, L., and Stylos, N., *Ber. deut. chem. Ges.*, **21**, 1144 (1888).
19. Fischer, H., and Fink, E., *Z. physiol. Chem.*, **280**, 123 (1944).
20. Boileau, J., *Bull. soc. chim. biol.*, **21**, 761 (1954).
21. Patton, S., and Kurtz, G. W., *J. Dairy Sci.*, **34**, 669 (1951).
22. Jennings, W. G., Dunkley, W. L., and Reiber, H. G., *Food Research*, **20**, 13 (1955).
23. Waravdekar, V. S., and Saslaw, L. D., *Biochim. et Biophys. Acta*, **24**, 439 (1957).
24. Sprinson, D. B., and Chargaff, E., *J. Biol. Chem.*, **164**, 433 (1946).
25. Huebner, C. F., Ames, S. R., and Bubl, E. C., *J. Am. Chem. Soc.*, **68**, 1621, (1946).
26. Mitsuhashi, S., and Davis, B. D., *Biochim. et Biophys. Acta*, **15**, 54 (1954).
27. Bandurski, R. S., and Greiner, C. M., *J. Biol. Chem.*, **204**, 781 (1953).
28. Tchen, T. T., Loewus, F. A., and Vennesland, B., *J. Biol. Chem.*, **213**, 547 (1955).
29. Rose, I. A., and Rieder, S. V., *J. Am. Chem. Soc.*, **77**, 5764 (1955); *J. Biol. Chem.*, **231**, 315 (1958).
30. Bloom, B., and Topper, Y. J., *Science*, **124**, 982 (1956).
31. Topper, Y. J., Mehler, A. H., and Bloom, B., *Science*, **126**, 1287 (1957).
32. Comb, D. G., and Roseman, S., *J. Am. Chem. Soc.*, **80**, 497 (1958).
33. Burton, K., and Krebs, H. A., *Biochem. J.*, **54**, 94 (1953).
34. Lehninger, A. L., Sice, J., and Jensen, E. V., *Biochim. et Biophys. Acta*, **17**, 285 (1955).
35. Kovachevich, R., and Wood, W. A., *J. Biol. Chem.*, **213**, 757 (1955).

Metabolism of the Retina

I. RESPIRATION OF CATTLE RETINA*

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In view of the many interesting metabolic properties of the retina it is surprising to find that relatively few investigations on this subject have been reported. The retina is reputed to have high rates of respiration and glycolysis as well as a strong Pasteur effect (1). In this paper observations on the metabolism of the retina dealing with various aspects of respiration are reported. An attempt was made to identify the endogenous substrate capable of sustaining the respiration of retinal tissue. This led to a study of the cofactors and substrates capable of stimulating the respiration of retinal homogenates. In the course of these studies it was possible to obtain evidence indicating the presence of the tricarboxylic acid cycle and the phosphogluconate oxidative pathway in this ocular tissue.

EXPERIMENTAL

Lactic acid was determined by the method of Barker and Summerson (2), hexose by the anthrone method (3), and α-keto-acids by the Friedemann and Haugen method (4). Oxygen consumption was determined in the Warburg apparatus with air as the gas phase and NaOH in the center well. To determine CO_2 production additional flasks were employed, which lacked alkali in the center wells, but contained H_2SO_4 in the side arms. At the end of the incubation period the acid was tipped in, and the CO_2 produced was calculated from the gas evolved, after correction for that present at the beginning of the incubation period. For the calculation it was assumed that oxygen consumption was not altered in flasks which lacked alkali in the center wells to trap the CO_2 formed. The methods used for the incubation of C^{14}-glucose, and recovery and analysis of $C^{14}O_2$ and C^{14}-lactate have been described (5). Cattle eyes were obtained from a local slaughter house.

RESULTS

As shown in Table I, the Q_{O_2} of the intact retina was about 10 and decreased gradually with time. The addition of glucose to the incubation medium did not elevate the O_2 consumption nor did it sustain the rate. It appeared that the respiration was maintained by the presence of endogenous substrate in the retina. Even in homogenates the Q_{O_2} was maintained nearly as high as that of the intact tissue for about 1 hour. With added glucose, the Q_{O_2} of retinal homogenates was stimulated somewhat and did not drop off as markedly as it did without the added substrate. To obtain information on the optimal conditions for the respiration of retinal homogenates, a study was made of the cofactors capable of stimulating respiration and aerobic glycolysis in retinal homogenates supplemented with glucose. It was found that even without any added cofactors retinal homogenates consumed oxygen at nearly half the rate of fortified preparations and, in addition, were able to accumulate some lactic acid (Table II). With fortified homogenates a Q_{O_2} of 14 was frequently obtained during the 1st hour, whereas the intact tissue generally gave a Q_{O_2} of about 10. The presence of all of the cofactors appeared to be necessary for a maximal rate of respiration. Lactic acid formation was depressed by the omission of either $MgSO_4$ or ATP, but not by the omission of DPN.

Although the high endogenous respiration in other tissues (6) has sometimes been attributed to the utilization of glycogen, or of fatty acids (7), R.Q.'s approaching 1 have been reported in studies of the retina (8), and the glycogen content of cattle retina has been reported to be only 92 mg./100 gm. of tissue (9). This level amounts to 2.8 μmoles of glycogen per retina (50 mg. of dry weight) and thus could account for only about 50 per cent of the oxygen consumed in 3 hours by the intact tissue. To demonstrate experimentally that glucose or glycogen was not the principal endogenous substrate supporting respiration, a study of the effect of iodoacetate was undertaken (Table III). It was not possible to find a concentration of iodoacetate which would block glycolysis completely without showing some inhibition of lactate utilization. However, it was possible to show for the intact tissue and retinal homogenates that during the 1st hour of incubation endogenous respiration was inhibited only about 15 per cent by iodoacetate at a concentration that suppressed glycolysis almost completely. Thus during the 1st hour of incubation respiration could be supported by endogenous substrate even in the absence of an active glycolysis. Noteworthy in these experiments (Table III) is the high capacity of the retina for aerobic glycolysis. This is illustrated by the accumulation of large amounts of lactic acid when glucose is added to the medium and demonstrates the fact that the glycolytic rate of this tissue far exceeds the rate at which its tricarboxylic acid cycle operates.

The possibility that the phosphogluconate oxidation pathway might be contributing to the endogenous respiration of the retina was then considered. In view of the report by Eichel (10)

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TABLE I

Respiration of cattle retina

Flasks containing 2 μmoles of DPN, 0.02 μmole of cytochrome *c*, 110 μmoles of phosphate buffer of pH 7.4, glucose as indicated, and either a whole retina or homogenate equivalent to one retina (50 mg. of dry weight) in a final volume of 3.0 ml. were incubated at 37°. Each value reported is the mean of four observations.

Tissue	Glucose, 100 μmoles	Q_{O_2}		
		1st hour	2nd hour	3rd hour
		μl./hr./mg. dry weight		
Whole retina		10.6 ± 0.4	8.4 ± 0.8	6.0 ± 0.9
Whole retina	+	10.5 ± 0.7	9.7 ± 1.0	8.1 ± 1.6
Homogenate		9.1 ± 0.9	4.8 ± 0.8	2.0 ± 0.3
Homogenate	+	10.3 ± 1.0	8.0 ± 0.8	6.8 ± 1.0

TABLE II

Effect of supplements on respiration of cattle retina homogenates

Each flask contained homogenate equivalent to 11.7 mg. of dry weight, 30 μmoles of glucose, 110 μmoles of phosphate buffer of pH 7.4, and supplements as indicated in a final volume of 3.0 ml. Flasks were incubated at 37°. The supplement mixture contained 9 μmoles of $MgSO_4$, 6 μmoles of DPN, 6 μmoles of ATP, and 0.12 μmole of cytochrome *c*.

Additions	Q_{O_2}				Lactic acid formed in 4 hours
	1st hour	2nd hour	3rd hour	4th hour	
					μmoles
Supplement mixture	14.3	13.8	9.4	5.0	27.5
Without $MgSO_4$	12.4	8.8	5.3	5.2	8.8
Without DPN	9.2	6.2	4.8	3.7	29.7
Without ATP	9.6	8.2	6.8	6.2	10.2
Without cytochrome *c*	8.7	7.5	3.8	2.4	28.6
With no supplements	6.4	5.3	4.2	3.0	8.3

TABLE III

Effect of inhibitors on respiration of cattle retina

Flasks containing either one retina (50 mg. of dry weight), 110 μmoles of phosphate buffer of pH 7.4, and additions as indicated in a final volume of 3.0 ml., or retinal homogenate equivalent to 17 mg. dry weight, 9 μmoles of $MgSO_4$, 6 μmoles of DPN, 6 μmoles of ATP, 0.12 μmole of cytochrome *c*, 110 μmoles of phosphate buffer of pH 7.4, and additions as indicated in a final volume of 3.0 ml. were incubated at 37°.

Tissue	Additions			Q_{O_2}			Lactic acid found after 3 hours
	Glucose	Iodoacetate	Lactate	1st hour	2nd hour	3rd hour	
	μmoles	*μmoles*	*μmoles*				*μmoles*
Whole retina	0	0	0	10.3	9.1	5.9	0.67
	0	15	0	8.6	2.1	0.8	0.98
	100	0	0	9.8	8.3	6.7	98.3
	100	15	0	8.2	2.0	0.6	1.2
Homogenate	0	0	0	10.5	5.2	3.2	0.52
	0	0.7	0	9.3	3.0	1.4	0.34
	0	0	30	11.8	11.6	11.0	23.7
	0	0.7	30	11.0	9.6	8.7	24.1
	30	0	0	13.7	11.4	8.1	33.4
	30	0.7	0	10.2	5.9	2.9	1.2

TABLE IV

C^{14}-glucose metabolism of cattle retina

Cattle retina (50 mg., dry weight) was incubated in 8.0 ml. of modified Krebs-Ringer buffer of pH 7.4 for 1 hour at 38°. The values are corrected to an initial specific activity of the 1-C^{14}-glucose which was 350 c.p.m. per μmole. The values are reported as the mean of four observations.

Substrate	Gas phase	Glucose utilized	Lactate produced		Pyruvate added	$C^{14}O_2$
		μmoles	*μmoles*	*c.p.m.*	*μmoles*	*c.p.m.*
1-C^{14}-glucose	Air	48	84	14,400	0	406
6-C^{14}-glucose	Air	46	87	16,125	0	347
1-C^{14}-glucose	N_2				0	34
1-C^{14}-glucose	N_2				160	520
6-C^{14}-glucose	N_2				0	25
6-C^{14}-glucose					160	35

indicating the presence of a high concentration of glucose 6-phosphate dehydrogenase in the retina, it seemed worthwhile to establish whether the operation of this pathway might account for the respiration occurring in the presence of iodoacetate. To determine the significance of the phosphogluconate oxidation pathway in the aerobic metabolism of glucose, intact retinas were incubated with either glucose-1-C^{14} or glucose-6-C^{14}. As shown in Table IV, no evidence of any significant preferential oxidation of the carbon 1 (C-1) atom of glucose was observed in the retina, unlike other ocular tissues (11, 12). The rate at which the C-1 and C-6 of glucose was oxidized to CO_2 was about the same, as indicated by the C-1 to C-6 ratio of 1.2. Furthermore, the amount of C^{14} incorporation into lactic acid was about the same from either 1-C^{14}- or 6-C^{14}-glucose. These results strongly suggest that the phosphogluconate oxidation pathway does not play a significant role in the utilization of glucose by the retina. Although the phosphogluconate oxidation pathway does not appear to play an active role, it is possible to obtain evidence suggesting the presence of this pathway in the retina. In those tissues which possess a lactic dehydrogenase capable of reacting to some extent with TPN it is possible to couple the oxidation of 1-C^{14}-glucose to $C^{14}O_2$ by the dehydrogenases of the phosphogluconate pathway to the reduction of pyruvate (13). In an atmosphere of nitrogen the retina was capable of stimulating the oxidation of 1-C^{14}-glucose with pyruvate. As shown in Table IV, in the absence of pyruvate there occurs only a small amount of 1-C^{14}-glucose oxidation, but the addition of pyruvate in the medium markedly stimulates the C-1 oxidation. No stimulation of C-6 oxidation of glucose was observed under the same conditions. It is interesting to note that the corneal epithelium and retina, known for their high content of lactic dehydrogenase, are the tissues in which the coupling of the dehydrogenases of the glycolytic mechanism and the phosphogluconate pathway is most easily demonstrated.

The observations made thus far seemed to rule out glucose or glycogen as the principal endogenous substrate supporting respiration in the presence of iodoacetate. It seemed likely that lactic acid might be contributing significantly to endogenous respiration. An attempt was made to relate changes in the initial quantities of lactic acid and hexose to the amounts of pyruvate and CO_2 produced in retinal homogenates incubated for 1 hour. Initially cattle retinas contain from 1 to 3 μmoles

TABLE V

Utilization of endogenous substrate by cattle retina

Flasks containing 2 μmoles of DPN, 0.02 μmole of cytochrome *c*, 110 μmoles of phosphate buffer of pH 7.4, retinal homogenate equivalent to 46 mg. of dry weight, and inhibitors as indicated in a final volume of 3.0 ml. were incubated at 37° for 1 hour. Initial concentrations of lactate, hexose, and pyruvate, expressed as μ atoms of carbon, were, respectively, 17.3, 16.1, and 0.3. The results are the mean of four observations.

Inhibitors	μ atoms of carbon utilized			μ atoms of carbon formed			μ atoms of oxygen consumed		Per cent accounted for by products formed
	Lactate	Glucose	Total	Pyruvate*	CO_2	Total	Observed	Expected†	
None	−9.6	−3.6	−13.2	+6.0	+9.8	+15.8	14.6	10.9	75
Iodoacetate (15 μmoles)	−12.0		−12.0	+6.1	+6.7	+12.8	9.7	7.7	79
Arsenite (12 μmoles)	−9.4	−4.9	−14.3	+12.8	+5.9	+18.7	10.0	8.0	80

* The α-keto acid accumulating in the presence of arsenite was converted to the 2,4-dinitrophenylhydrazone. The absorption spectrum was identical with that of the derivative prepared from pyruvic acid. The 2,4-dinitrophenylhydrazone of the reaction product and of pyruvic acid each migrated at the same rate in 1 per cent Na_2CO_3, *n*-butanol:ethanol:H_2O (50:10:40) by volume, phenol saturated with water, and 3 per cent NH_3.

† The expected oxygen consumption was calculated from the quantities of pyruvate and CO_2 formed, on the assumption that these compounds arose from lactate or hexose.

TABLE VI

Effect of various substrates on retinal respiration

Cattle retina homogenate equivalent to 11.5 mg. of dry weight was incubated for 1 hour at 37° in flasks containing 9 μmoles of $MgSO_4$, 6 μmoles of DPN, 6 μmoles of ATP, 0.12 μmole of cytochrome *c*, 110 μmoles of phosphate buffer of pH 7.4, and 30 μmoles of substrate as indicated.

Substrate	Q_{O_2}			Substrate	Q_{O_2}		
	1st hour	2nd hour	3rd hour		1st hour	2nd hour	3rd hour
None	8.0	4.7	3.7	None	5.7	4.0	3.0
Glucose	12.8	10.1	8.2	Glucose	11.1	8.3	7.9
L-Lactate	14.9	9.0	7.6	α-Ketoglutarate	10.7	7.5	4.6
Pyruvate	10.3	8.0	7.7	Succinate	13.4	7.4	4.8
Citrate	14.4	10.0	7.5	Fumarate	9.5	6.5	5.0
DL-Isocitrate	12.7	6.5	4.8	L-Malate	11.9	7.0	5.9

of hexose per retina (50 mg., dry weight) and from 4 to 5 μmoles of lactic acid. The results, expressed as μ atoms of carbon, Table V, indicated that considerably more lactic acid was utilized than hexose. Together the quantities of lactate and hexose utilized were sufficient to account for the major portion of the pyruvate and CO_2 formed. These products accounted for about 75 per cent of the oxygen consumed. In the presence of iodoacetate lactic acid utilization was unimpaired; no hexose was utilized. In this experiment the amount of CO_2 and pyruvate produced was equivalent to the lactic acid consumed and sufficient to account for nearly 80 per cent of the oxygen utilized. In the presence of arsenite considerably more pyruvate accumulated than in its absence. No α-ketoglutarate accumulated, although retinal tissue is reported to contain 1.83 mg. of glutamic acid per gm. of tissue (14), amounting to 2.9 μmoles per cattle retina, and has been shown to accumulate α-ketoglutarate when incubated with glutamic acid in the presence of arsenite (15). These experiments suggest lactate to be a major endogenous substrate contributing to respiration in the presence of iodoacetate and indicate that at least 25 per cent of the observed endogenous respiration cannot be accounted for by the utilization of hexose or lactate.

The fact that lactic acid appeared to be an important endogenous substrate suggested that an active tricarboxylic acid cycle was functioning in the retina. The ability of the intermediates of the tricarboxylic acid cycle to stimulate oxygen consumption in retinal homogenates, as shown in Table VI, supports the view that the tricarboxylic acid cycle is operating in this tissue. A more detailed study of the tricarboxylic acid cycle in retinal mitochondria is now being undertaken.

DISCUSSION

The very high rate of respiration, (Q_{O_2} = 31) that has been attributed to the retina is based on Warburg's data (16) in which various rat tissue slices including retina, were compared. However, data compiled by deBerardinis (17) indicate considerable variation between species in the rate of oxygen consumption of the retina. In our experiments the Q_{O_2} of cattle retinas was approximately 10 which is within the range previously reported by Greig and Munro (18). It should be noted that in these studies from 1 to 2 hours elapsed between the removal of the cattle eyes at the slaughter house and the addition of retinal tissue to the flasks. However, retinas from eyes stored for 2 hours consumed oxygen at about the same rate as those used after 1 hour. Moreover, experiments in which the Q_{O_2} was determined for rabbit retinas, removed immediately after death and also 1 hour thereafter, indicated that there was no significant decrease in the respiratory capacity of the retina upon brief storage in the intact eye.

The outstanding metabolic property of the retina seems to be

its capacity for aerobic glycolysis. In these studies *in vitro*, the aerobic oxidative mechanisms of the cattle retina cannot keep pace with the glycolysis and consequently lactic acid accumulates. In view of these results, it seems somewhat surprising that the retina *in situ* does not have a higher concentration of lactic acid. Adequate supplies of glucose must be available to this tissue since it is vascularized. Yet one is left with the impression that only a small fraction of the total capacity for glycolysis is ever utilized. Possibly lactate is actually formed at a much higher rate than is apparent, but diffusion into surrounding tissues never allows it to reach an unfavorable level. If this is so, the diffusion of lactate is probably in the direction of the choroid and not into the vitreous humor since only a small amount of lactate is found in the latter tissue. The existence of a regulatory mechanism is a more attractive possibility. There may be some control of the glycolytic mechanism *in vivo* which is not operative under conditions *in vitro*. These questions on glycolysis and the important relationship to the Pasteur effect are problems currently being investigated.

SUMMARY

The utilization of endogenous lactic acid and hexose appears to contribute significantly to the endogenous respiration of cattle retina homogenates. Tricarboxylic acid cycle substrates increase the oxygen consumption of homogenates.

The utilization of glucose by the retina appears to occur almost exclusively through glycolysis and the tricarboxylic acid cycle, although under suitable conditions the hexose monophosphate shunt pathway can be activated.

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REFERENCES

1. Pirie, A., and Van Heyningen, R., *Biochemistry of the eye*, Blackwell Scientific Publications, Oxford, 1956, p. 205.
2. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941).
3. Scott, T. A., and Melvin, E. H., *Anal. Chem.*, **25**, 1656 (1953).
4. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
5. Kinoshita, J. H., *A. M. A. Arch. Ophthalmol.*, **54**, 360 (1955).
6. Marsh, M. E., *J. Nutrition*, **13**, 109 (1937).
7. Wenner, C. E., and Weinhouse, S., *J. Biol. Chem.*, **222**, 399 (1956).
8. Dickens, F., and Simer, F., *Biochem. J.*, **24**, 1301 (1930).
9. Crane, R. K., and Ball, E. G., *J. Biol. Chem.*, **188**, 819 (1951).
10. Eichel, B., School of Aviation Medicine, U. S. A. F., Report No. 56-31 (1956).
11. Kinoshita, J. H., and Wachtl, C., *J. Biol. Chem.*, **233**, 5 (1958).
12. Kinoshita, J. H., Masurat, T., and Helfant, M., *Science*, **122**, 72 (1955).
13. Kinoshita, J. H., *J. Biol. Chem.*, **228**, 247 (1957).
14. Pirie, A., and Van Heyningen, R., *Biochemistry of the eye*, Blackwell Scientific Publications, Oxford, 1956, p. 208.
15. deVincentiis, M. and Auricchio, G., *Acta Ophthalmol. Graefe's*, **28**, 7 (1950).
16. Warburg, O., *Biochem. Z.*, **184**, 484 (1927).
17. deBerardinis, E., *Arch. Ottalmol.*, **55**, 167 (1951).
18. Greig, M. E., and Munro, M. P., *Biochem. J.*, **33**, 143 (1939)

L-2-Keto-4,5-dihydroxyvaleric Acid: an Intermediate in the Oxidation of L-Arabinose by *Pseudomonas saccharophila*

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Crude cell-free preparations of *Pseudomonas saccharophila* grown on L-arabinose oxidize L-arabinose to α-ketoglutaric acid (1). L-Arabonic acid has been identified as an intermediate in this reaction. The investigations described in this communication are concerned with the mechanism of the conversion of L-arabonate to α-ketoglutarate. Evidence is presented for the intermediate formation of L-2-keto-4,5-dihydroxyvaleric acid, a 2-keto-3-deoxy sugar acid, in the transformation. In this respect the metabolism of L-arabinose is similar to that of the D-isomer which is dehydrated to D-2-keto-4,5-dihydroxyvaleric acid (2). The metabolism of D- and L-arabinose differs in that the D-form of the keto acid intermediate is oxidatively cleaved to pyruvic and glycolic acids while the L-isomer is converted to α-ketoglutaric acid. The over-all reaction sequence, as it is now known, in the metabolism of L-arabinose by *P. saccharophila* can be represented as follows:

$$\text{L-arabinose} \xrightarrow[\text{(DPN or TPN)}]{-2H} \text{L-arabono-}\gamma\text{-lactone} \xrightarrow{+H_2O}$$

$$\text{L-arabonate} \xrightarrow{-H_2O} \text{L-2-keto-4,5-dihydroxyvalerate} \xrightarrow[(DPN)]{-2H,\ -H_2O} \alpha\text{-ketoglutarate}$$

EXPERIMENTAL

Cultures of *P. saccharophila* were grown on L-arabinose as the sole carbon source, harvested, and stored as previously described (1). Cells have been stored at −20° for 2 years without loss of enzymatic activity. Cell-free preparations were obtained by grinding with alumina. The frozen cells were placed in a mortar chilled to approximately 5°, ground for 10 minutes with three parts of alumina (Alcoa A-301), and then extracted with 3 volumes of cold, 0.004 M phosphate buffer, pH 6.8. The suspension was centrifuged at 0° and 20,000 × *g* for 30 minutes. Streptomycin sulfate, 0.1 volume of 20 per cent, was added to the supernatant liquid in order to precipitate nucleic acids. The latter were removed by centrifugation and discarded. Little or no loss of protein or enzymatic activity occurred in this step. Because of the lability of the enzyme system, extracts were used the same day they were prepared.

L-Arabonic acid was assayed as the lactone (3) which was produced by heating the solution of the acid in 0.1 N HCl in a boiling water bath for 5 minutes. Formaldehyde was measured by its reaction with chromotropic acid (4). β-Formylpyruvic acid was determined by the colorimetric test for 2-keto-3-deoxy sugar acids described by Weissbach and Hurwitz[1] except that the time for oxidation by periodic acid was not permitted to exceed 5 minutes. Longer oxidative periods resulted in low yields.

The intermediate, L-2-keto-4,5-dihydroxyvaleric acid, was estimated enzymatically by measuring DPN reduction. The intermediate, 0.5 μmole or less, in 0.1 ml. was mixed with 0.05 ml. of crude enzyme extract, 0.1 ml. of 0.01 M DPN, 0.1 ml. of 0.02 M EDTA[2] (or 0.005 M Na_2S), and 2.65 ml. of 0.1 M Tris buffer, pH 8.0. Reduction of DPN was determined spectrophotometrically at 340 mμ. Mg^{++} stimulated the rate at which DPN was reduced by L-arabonate but had no effect on the rate when the intermediate was the substrate. Thus, interference by L-arabonate in the assay was minimized by the omission of Mg^{++}.

For certain purposes, 2-keto-4,5-dihydroxyvaleric acid was qualitatively detected by mixing 1 drop of a solution with 2 drops of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl. After 5 minutes the solution was made alkaline. The appearance of a reddish-brown color indicated the presence of the compound. α-Ketoglutaric acid was measured by the method of Friedemann and Haugen (5). 2-Keto-4,5-dihydroxyvaleric acid, when present in equimolar amounts with α-ketoglutaric acid, did not cause more than a 10 per cent error in the measurement.

For paper chromatographic procedures, the solvents used were: Solvent 1, ether:benzene:formic acid:water (70:30:14:10); Solvent 2, *sec*-butanol (washed with $FeSO_4$ to remove peroxides and then with water):formic acid (95:5); Solvent 3, *n*-propanol:formic acid:water (6:3:1); Solvent 4, methyl cellosolve:water:concentrated NH_4OH (80:15:5); Solvent 5, *n*-butanol:pyridine:water (6:4:3). Acids were detected by spraying with bromthymol blue (6); α-keto acids were detected with a semicarbazide spray (7); and lactones were located with a reagent described by Abdel-Akher and Smith (8). The 2,4-dinitrophenylhydrazones were prepared for chromatography as described by Cavallini (9). The nitroquinoxaline derivatives were prepared as described by Hockenhull and Foodgate (10).

L-Arabonic acid was prepared by the hypoiodite oxidation of

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[1] A. Weissbach and J. Hurwitz, personal communication.

[2] The abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

L-arabinose (11). 2,4,5-Trihydroxyvaleric acid, a reduced product of 2-keto-4,5-dihydroxyvaleric acid, was synthesized as described by Nef (12).

The methods of synthesizing 3,4-dihydroxybutyric acid (the expected product of oxidative decarboxylation of 2-keto-4,5-dihydroxyvaleric acid) described by Glattfeld *et al.* (13) and Glattfeld and Rietz (14) were abandoned because of unsatisfactory yields. Yields approaching 100 per cent were obtained in the following procedure: 25 gm. (0.37 mole) of 3-butenenitrile are dissolved in 250 ml. of 90 per cent formic acid to which are added 45 ml. of 30 per cent H_2O_2 (0.4 mole). The reaction mixture is allowed to stand in a 37° water bath for 24 hours and finally heated on a steam bath for 2 hours to hydrolyze the nitrile completely to the acid. Water and formic acid are removed by distillation under reduced pressure and the residue is redissolved in 100 ml. of water and passed through a Dowex 50 (H^+) column. The eluate is distilled in a vacuum and the fraction boiling from 155° to 158° at 5 mm. is collected. The yield of lactone is about 35 gm. or 92.5 per cent.

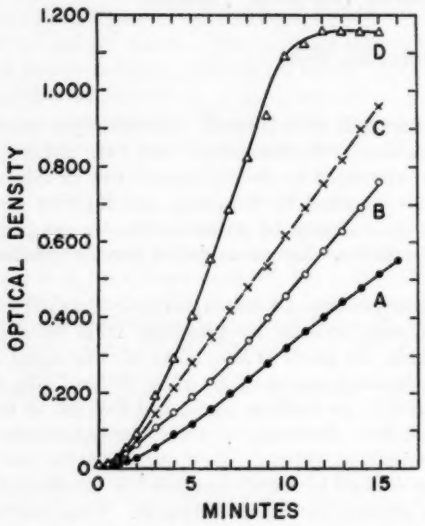

FIG. 1. The effect of Mg^{++} and S^{--} on the rate of reduction of DPN. *Curve A*, 0.1 ml. of enzyme was mixed with 2.5 ml. of 0.10 M Tris, pH 8.0, 0.1 ml. of 0.10 M potassium L-arabonate, 0.1 ml. of 0.005 M DPN and water to bring the volume to 3.0 ml. Substrate was added at zero time; *Curve B*, same as *Curve A* except that 0.1 ml. of 0.005 M Na_2S was added; *Curve C*, Same as *Curve A* except that 0.1 ml. of 0.05 M $MgCl_2$ was added; *Curve D*, Same as *Curve A* except that both 0.1 ml. of 0.005 M Na_2S and 0.1 ml. of 0.05 M $MgCl_2$ were added. Optical density measurements were made at 340 mμ.

TABLE I

Determination of amount of active intermediate formed from potassium L-arabonate

Enzyme, 0.5 ml., was mixed with 0.15 ml. of 0.5 M potassium L-arabonate, 0.5 ml. of 0.5 M Tris or NH_4OH-NH_4Cl buffer, and 0.35 ml. of water. At the time intervals listed, 0.2 ml. of the reaction mixture was removed and diluted to 1.0 ml. with 0.1 M of Tris, pH 8.0. Then 0.5 ml. of the diluted solution was assayed for L-arabonate by the lactone test and 0.1 ml. was used to measure the intermediate by DPN reduction.

Length of preincubation period	Tris buffer, pH 8.0		Tris buffer, pH 9.0		NH_4OH buffer, pH 9.0	
	L-Arabonate	Active intermediate	L-Arabonate	Active intermediate	L-Arabonate	Active intermediate
min.	*μmoles/ml.*	*μmoles/ml.*	*μmoles/ml.*	*μmoles/ml.*	*μmoles/ml.*	*μmoles/ml.*
0	52.1	0	52.0	0	52.6	0
30	47.1	4.0	39.6	9.5	46.2	5.5
60	41.2	8.8	24.5	13.1	39.6	13.2
120	30.7	1.3	13.7	12.7	26.6	18.7
180			8.4	8.7	19.3	27.8

RESULTS

Formation of 2-Keto-4,5-dihydroxyvaleric Acid

Crude enzyme preparations from *P. saccharophila* reduce DPN with L-arabonate as substrate only after a definite lag period (1). If Na_2S and $MgCl_2$ are included in the reaction mixture, the time required for the quantitative reduction of DPN is significantly reduced and the lag period is shortened but not entirely abolished (Fig. 1). Other substances combining with ions of heavy metals, such as 2,3-dimercaptopropanol, cysteine, glutathione, and EDTA, can replace sulfide.

It has also been reported that an unstable intermediate is formed by a nonoxidative process when L-arabonate is incubated with crude extract in the absence of DPN (1). The presence of this substance was detected by the immediate and rapid reduction of DPN when DPN was added to the reaction mixture. In preliminary experiments, a method of assay was developed based on the enzymatic reduction of DPN in the presence of the intermediate. Evidence that this was a valid measure of the intermediate was obtained in subsequent experiments with the purified intermediate where the amount of DPN reduced corresponded to the amount of α-ketoglutaric acid produced in the oxidation (Table III). Using this assay, the formation and stability of the intermediate was investigated. The compound apparently is inactivated in some manner in Tris buffer (Table I). In NH_4Cl-NH_4OH buffer, the intermediate is more stable and there is a better correlation between the amount of L-arabonate consumed and the amount of intermediate formed. The optimum pH for formation of the intermediate in the ammonium buffer is 9; however, at higher pH's the compound is unstable in this buffer, also.

The addition of most deproteinizing agents to the reaction mixture resulted in a loss in enzymatic activity of the intermediate. The most satisfactory method of deproteinization was by acidification of the reaction mixture with Dowex 50 (H^+). The compound may be stored for several months under neutral or acid conditions if kept at −20°. At room temperature there was a slow loss of activity over a period of several days. Alkaline solutions of the intermediate became inactive within a few hours at room temperature. When heated, these alkaline solutions turned a bright reddish-orange color.

The first indication that the intermediate formed from L-arabonate is a keto compound was obtained when it was observed that there was a reaction between the compound and 2,4-dinitrophenylhydrazine. The intermediate also reacted with semicarbazide (15) and *o*-phenylenediamine (16) to produce addition products which have absorption maxima at 250 mμ and 325 mμ, respectively. The latter two tests are considered to be specific for α-keto acids.

Paper chromatographic examination indicated that only one intermediate accumulates in the reaction mixture. The reac-

tion mixture, after being treated with Dowex 50 (H^+), was applied to the chromatogram sheet and developed by ascending chromatography in one of the solvents listed in the section on "Methods." Location of the compound was detected by spraying the sheet with either an acid or an α-keto acid indicator. All chromatograms have shown the presence of only one compound in the reaction mixture other than the substrate, L-arabonate. In each of the five solvents the intermediate has an R_F different from either L-arabonate or α-ketoglutarate (Table II).

Isolation of 2-Keto-4,5-dihydroxyvaleric Acid

Isolation of the intermediate was accomplished by chromatography on Dowex 1 columns. A reaction mixture composed of 4.0 ml. of crude enzyme preparation, 2.0 ml. of 0.5 M potassium L-arabonate, 2.0 ml. of 0.02 M EDTA, 1.0 ml. of 0.05 M $MgSO_4$, and 1.0 ml. of 0.5 M NH_4OH-$(NH_4)_2SO_4$ buffer, pH 9.0, was incubated at 30° for 3 hours. The pH of the mixture, which decreased as the reaction proceeded, was readjusted to pH 9 with 1.0 M KOH at frequent intervals. Assay of the incubation mixture by the DPN reduction method indicated that 920 μmoles of 2-keto-4,5-dihydroxyvalerate had been formed. The reaction mixture, which contained a flocculent precipitate, was deproteinized by passing it through a column of Dowex 50 (H^+), and the column was washed with water until the effluent gave a negative test for α-keto acid (as determined by reaction with 2,4-dinitrophenylhydrazine). The effluent was then passed through a Dowex 1 (formate) column. Next, the column was washed thoroughly with 0.25 M formic acid which removed residual L-arabonate. The intermediate was then eluted from the column with 0.275 M formic acid. Fractions giving a positive α-keto acid test were pooled and concentrated by lyophilization. The residue was dissolved in water and assayed. At this point recovery of the enzymatically active intermediate was 90 per cent. Solvent could not be removed by distillation under reduced pressure without complete loss of activity.

The lyophilized material was a gummy syrup which showed no tendency to crystallize. It was soluble in water, ethanol, and acetone but insoluble in ether. Various salts were prepared by neutralization and lyophilization. These were obtained as syrups which showed the same solubility properties as the unneutralized intermediate.

Aqueous solutions of the lyophilized intermediate gave a positive ferric chloride test for the enol group. By chemical means to be discussed later, it was found that 80 to 90 per cent of this preparation existed in the lactone form and the remainder as the free acid. At pH 7, the lactone was completely hydrolyzed within 3 hours while only 5 minutes were required at pH 8. Chromatograms of the lactone showed the presence of a single spot characterized by an R_F which was different from that of L-arabono-γ-lactone (Table II). Chromatograms of the free acid, obtained by neutralizing and hydrolyzing the lactone and then passing the solution through a Dowex 50 (H^+) column, also revealed a single spot when sprayed with either an acid or α-keto acid indicator.

The intermediate was identified chromatographically by comparison with DL-2-keto-4,5-dihydroxyvaleric acid which has been chemically synthesized.[3] The R_F values of the free acid and lactone form of the intermediate are identical with those of the corresponding forms of the synthetic compound. In addition, both the intermediate and synthetic DL-2-keto-4,5-dihydroxyvaleric acid are enzymatically oxidized to α-ketoglutaric acid. While the intermediate is quantitatively oxidized in a reaction mixture of crude enzyme, DPN, EDTA, and Tris buffer, pH 8.0, 50 per cent of the synthetic keto acid is transformed which is the expected result for the DL-compound (Table III).

[3] Details of the synthesis of DL-2-keto-4,5-dihydroxyvaleric acid will be presented in a subsequent report.

TABLE II

Comparison of R_F values of synthetic compounds with enzymatically produced intermediate and its derivatives

	Solvents*				
	1	2	3	4	5
L-Arabonic acid	0.00	0.33	0.34	0.56	0.16
L-Arabono-γ-lactone		0.50	0.54	1.00	0.73
α-Ketoglutaric acid	0.40	0.71	0.86	0.50	0.21
2-Keto-4,5-dihydroxyvaleric acid	0.15	0.59	0.64	0.74	0.29
Intermediate	0.16	0.59	0.63	0.77	0.29
Lactone of 2-keto-4,5-dihydroxyvaleric acid†		0.71	0.75	1.00	0.82
Lactone of intermediate†		0.71	0.75	1.00	0.81
3,4-Dihydroxybutyric acid	0.11	0.64	0.62	0.64	0.34
Oxidized intermediate‡	0.11	0.64	0.60	0.60	0.30
Lactone of 3,4-dihydroxybutyric acid†		0.77	0.88	1.00	0.72
Lactone of oxidized intermediate†		0.78	0.83	1.00	0.72
2,4,5-Trihydroxyvaleric acid	0.03	0.54	0.32	0.68	0.21
Reduced intermediate§	0.03	0.57	0.30	0.66	0.19
Lactone of 2,4,5-trihydroxyvaleric acid†		0.61	0.65	1.00	0.72
Lactone of reduced intermediate†		0.67	0.65	1.00	0.75

* See "Methods" for composition of the solvents.

† Lactones were prepared by heating a 0.1 M solution of the compound to 0.1 N HCl for 5 minutes in a boiling water bath.

‡ Prepared by treating the intermediate with H_2O_2.

§ Prepared by treating the intermediate with sodium borohydride.

TABLE III

Comparison of various methods of assay for DL-2-keto-4,5-dihydroxyvaleric acid and enzymatically produced intermediate in aqueous solution

Substrate	Concentration of substrate* as determined by:		
	DPN reduction†	α-Ketoglutaric acid determination‡	Decarboxylation with hydrogen peroxide§
	μmoles/ml.	μmoles/ml.	μmoles/ml.
DL-2-Keto-4,5-dihydroxyvaleric acid	18.7	19.9	36.4
Intermediate	55.5	56.6	55.7

* Lyophilized preparations of the substrates were dissolved in arbitrary amounts of water and assayed by the methods listed.

† Dilutions of the aqueous solution were made so that a 0.1-ml. aliquot of the diluted sample, when added to a cuvette containing the assay mixture, would result in the reduction of 0.5 μmole or less of DPN.

‡ Reaction mixtures, used to measure DPN reduction, were pooled, and 2,4-dinitrophenylhydrazine added. The derivative was extracted and measured colorimetrically.

§ A volume of solution containing 2 to 5 μmoles of substrate (as determined by DPN reduction) was treated with H_2O_2 and CO_2 production measured as described in text.

Attempts to isolate the intermediate from deproteinized reaction mixtures as the calcium salt following the procedure described by Palleroni and Doudoroff (17) for the precipitation of the calcium salt of D-2-keto-4,5-dihydroxyvaleric acid were unsuccessful.

Chemical Properties

Oxidation by Ceric Sulfate and Hydrogen Peroxide—Ceric sulfate is an oxidizing agent that is usually specific for α-keto acids if the reaction is measured by CO_2 production. However, it could not be used for the decarboxylation of 2-keto-4,5-dihydroxyvaleric acid since it caused extensive degradation of the compound resulting in the production of large amounts of CO_2.

Hydrogen peroxide will also decarboxylate α-keto acids, and has the advantage over ceric sulfate that strongly acidic conditions are not required. It was found that hydrogen peroxide produced exactly 1 mole of CO_2 per mole of intermediate under the following conditions: 5 μmoles of the intermediate were placed in a Warburg flask with two side arms. An excess (0.2 ml.) of 0.1 N KOH was added and the volume was brought to 1.0 ml. with water. In one side arm was placed 0.1 ml. of 0.5 N acetic acid and in the other 0.1 ml. of 3 per cent H_2O_2. After temperature equilibration, the acetic acid was tipped into the flask. After another 10 minutes, peroxide was added. Decarboxylation was usually complete within 5 to 10 minutes.

Since peroxide will not react with the lactone, it is necessary to hydrolyze the lactone ring by the addition of excess base. Acetic acid was then used to acidify the reaction mixture since acetate buffer is most efficient at pH 4. At this pH, (*a*) the intermediate does not lactonize, (*b*) the solubility of CO_2 is negligible, and (*c*) hydrogen peroxide does not spontaneously decompose to oxygen and water.

In another experiment, 100 μmoles of the intermediate were decarboxylated with hydrogen peroxide. When the oxidation was complete, the reaction mixture was passed through a Dowex 1 (formate) column. The column was washed with water and the decarboxylated compound then eluted with 0.1 N HCOOH. It was detected in the eluate by the lactone test. However, the compound did not react with 2,4-dinitrophenylhydrazine. Fractions giving a positive lactone test were pooled and lyophilized. The residue was dissolved in water, a known excess of NaOH added, and the solution back-titrated to the phenolphthalein end point. The lyophilized eluate contained 92 μeq. of acid. Chromatography of the eluted compound on paper as the free acid and as the lactone revealed the presence of a single compound possessing an R_F indistinguishable from that of the corresponding forms of 3,4-dihydroxybutyric acid (Table II). It was also found that both 3,4-dihydroxybutyrate and the compound formed by the decarboxylation of the intermediate could be oxidized by periodate with the production of 1 μmole of formaldehyde per μmole of substrate.

These results demonstrate that the intermediate is oxidized by hydrogen peroxide to CO_2 and 3,4-dihydroxybutyric acid. Comparable results were obtained with a known sample of DL-2-keto-4,5-dihydroxyvaleric acid.

This reaction furnishes a convenient method for measuring 2-keto-4,5-dihydroxyvaleric acid. By omitting the hydrolysis step in the assay described above, it is possible to measure the amount of free acid in the presence of the lactone.

Periodic Acid Oxidation—The course of oxidation of 2-keto-4,5-dihydroxyvaleric acid, of either enzymatic or chemical origin, by periodate was followed by assaying for formaldehyde and β-formylpyruvate. Since periodate will not oxidize the lactone form of the intermediate, the lactone was hydrolyzed with base and then the required amount of periodate was added along with enough H_2SO_4 to give a final concentration of 0.1 N acid. A μmole of formaldehyde was produced per μmole of substrate within 5 minutes, and the amount did not significantly change in the next 120 minutes (Table IV).

The maximal quantity of β-formylpyruvate was obtained 5 minutes after mixing the intermediate with periodate (Table IV). Its concentration then decreased until it had almost disappeared at the end of 60 minutes. The spectrum of the color produced had an absorption maximum at 550 mμ. The results are given in optical density units since an authentic sample of formylpyruvate was not available for molar absorption determinations.

The disappearance of formylpyruvate very likely was due to its decarboxylation by periodate. In a separate experiment in which the oxidation was performed in a Warburg respirometer at 30°, it was found that 1 μmole of CO_2 was produced in 60 minutes from 1 μmole of 2-keto-4,5-dihydroxyvaleric acid. No steam volatile acids could be detected in the experiment described in Table IV or in similar experiments performed on a larger scale.

The amount of periodate consumed could not be quantitatively measured by iodine titration because the oxidized products reacted slowly with the iodine resulting in false and fading end points. Also, it was not possible to measure periodate reduction spectrophotometrically since the products of the reaction had larger extinction coefficients than the periodate.

These results are consistent with the identification of the intermediate of L-arabinose metabolism by *P. saccharophila* as 2-keto-4,5-dihydroxyvaleric acid. The fact that the compound gives a positive reaction with 2-thiobarbituric acid after being oxidized by periodate establishes it as a 2-keto-3-deoxy sugar acid. The simultaneous formation of formaldehyde and β-formylpyruvate without the concomitant production of formic acid from this oxidation can be obtained with only one 2-keto-3-deoxy sugar acid, 2-keto-4,5-dihydroxyvaleric acid.

TABLE IV

Determination of formaldehyde and β-formylpyruvic acid produced by oxidation of L-2-keto-4,5-dihydroxyvaleric acid by periodate

A volume of 0.3 ml. of 0.1 N NaOH was added to 0.2 ml. of a solution of 2-keto-4,5-dihydroxyvaleric acid containing 55.5 μmoles per ml. in order to hydrolyze the lactone. After 5 minutes 0.1 ml. of 1 N H_2SO_4 and 0.5 ml. of 0.1 M HIO_4 were added. The reaction mixture was incubated at room temperature. At the time intervals listed, 0.1 ml. was transferred to a solution of 1 ml. of 1 N H_2SO_4 and 1 ml. of 1 N Na_3AsO_3. After the iodine color had faded, the solution was diluted to 5 ml. and assayed for formaldehyde and β-formylpyruvic acid.

Time	Formaldehyde	β-Formylpyruvic acid
minutes	*total μmoles*	*optical density units*
0	0	0
5	10.9	1.810
10	10.8	0.830
20	11.2	0.340
30	11.4	0.225
60	12.0	0.090
120	12.5	0.000

Reaction with 2,4-Dinitrophenylhydrazine and 4-Nitro-o-phenylenediamine—Although it was demonstrated that the intermediate which had been treated with alkali in order to hydrolyze the lactone contained a carbonyl group adjacent to the carboxyl, it was not possible to obtain a solid derivative using the usual reagents for α-keto acids. Both 2,4-dinitrophenylhydrazine and 4-nitro-o-phenylenediamine reacted with the intermediate as could be shown by the intense color produced when the reaction mixtures were made alkaline. However, the derivatives did not precipitate from acid solution. Attempts to purify the derivatives by extraction from acid solution into ethyl acetate followed by removal of the derivative from the ethyl acetate by washing with 10 per cent Na_2CO_3 were unsuccessful. In contrast, Palleroni and Doudoroff (17) were able to obtain a solid 2,4-dinitrophenylhydrazone of the D-isomer of the α-keto acid which could be dissolved in ethyl acetate and recrystallized from ethanol.

Reduction with Sodium Borohydride—An aqueous solution, 5 ml., of 0.04 M intermediate was neutralized with NaOH until the pH remained at 8, and 100 mg. of solid sodium borohydride were added. Reduction was complete within 5 minutes as determined by the absence of a reaction with 2,4-dinitrophenylhydrazine. After 15 minutes the excess borohydride was destroyed by acidifying with HCl to approximately pH 1 and then the reaction mixture was diluted to 10 ml. with water. A volume of 0.1 ml. was removed and oxidized by periodate; 1.8 μmoles of formaldehyde were produced in the oxidation but no CO_2. Also, no CO_2 was produced when a 1-ml. sample was treated with hydrogen peroxide (with the use of the procedure described for decarboxylating the intermediate).

To separate the reduced derivative from HCl and borate, it was converted into the lactone by heating for 5 minutes in a boiling water bath. After cooling to room temperature, the solution was passed through a Dowex 1 (formate) column. The lactone is not adsorbed by the resin. The eluate containing the reduced intermediate was neutralized to pH 8 to 9 in order to hydrolyze the lactone and the solution was again passed through a Dowex 1 (formate) column. The reduced intermediate, as a salt, is adsorbed by the column. After washing the column with 0.025 M formic acid, the compound was eluted with 0.03 M formic acid. The presence of the derivative in fractions of the eluate was detected by the lactone test. Fractions giving a positive test were pooled, lyophilized, and redissolved in water. The recovery of lactonizable material was 80 per cent. When chromatographed as the acid or as the lactone, the reduced intermediate possessed R_F values corresponding to those of a known sample of 2,4,5-trihydroxyvaleric acid or its lactone (Table II).

DISCUSSION

There are certain similarities in the metabolism of D-glucose (14, 18), D-galactose (19), and D-arabinose (2, 17) by *P. saccharophila*. The sequence of events is as follows. The sugar is oxidized to the aldonic acid which is then dehydrated to form the 2-keto-3-deoxy sugar acid. This latter compound is cleaved with the formation of pyruvic acid from the first three carbons. Details of the mechanism are different for different sugars. For example, glucose and all of its intermediates are phosphorylated. On the other hand, a phosphorylated compound does not appear in the pathway of galactose metabolism until just before the cleavage step. No phosphorylated compound appears to be formed in D-arabinose oxidation. With D-arabinose, the pattern also varies in that an oxidation occurs along with the cleavage of the 2-keto-3-deoxy sugar acid to form pyruvic and glycolic acids.

The oxidation of L-arabinose in *P. saccharophila* follows the same general metabolic scheme, except that α-ketoglutaric acid is formed rather than pyruvic acid (1). It has now been shown that L-arabonic acid may be dehydrated to a 2-keto-3-deoxy sugar acid. The identification of this compound is based on a comparison of its chemical properties with those of a known sample of 2-keto-4,5-dihydroxyvaleric acid, and by its reduction by sodium borohydride to a compound chromatographically identified as 2,4,5-trihydroxyvaleric acid.

The configuration of the hydroxyl group on the C_4 of the intermediate has not been determined. Since this carbon does not appear to be involved in the dehydration step, it probably remains in the L-configuration. This assumption is based on the stereospecificity of the enzymes involved in the dehydration of 6-phosphogluconic acid and of galactonic acid, and the subsequent cleavage of the dehydrated sugar acids (19). In the metabolism of these sugars, the configuration about the C_4 remains unchanged in the dehydration step. Comparable results have been obtained in L-arabinose metabolism. The dehydrase in the crude enzyme preparation is active on L-arabonate but not on D-arabonate (1). Also, only 50 per cent of DL-2-keto-4,5-dihydroxyvaleric acid are oxidized to α-ketoglutaric acid by the enzyme system.

A minimum of two steps would be required for the enzymatic oxidation of L-2-keto-4,5-dihydroxyvaleric acid to α-keloglutaric acid. It could occur either by a dehydration between carbons-4 and -5 followed by an oxidation, or the oxidation could be the first step and followed by a rearrangement. There is no direct evidence available to indicate which of these two schemes, or perhaps even another, is operating in *P. saccharophila*. It appears likely, however, that the oxidation is the first step. In reaction mixtures in which L-arabonate is the substrate but which lack DPN, no new compounds other than 2-keto-4,5-dihydroxyvaleric acid have been detected. Also, there is no lag or induction period in DPN reduction when 2-keto-4,5-dihydroxyvalerate is being oxidized to α-ketoglutarate. An induction period would be expected if the 2-keto-3-deoxy sugar acid first had to be converted to another compound for the oxidation to take place.

The only other report on 2-keto-4,5-dihydroxyvaleric acid is its biological formation from D-arabonate (2). While there are a few discrepancies, the properties described for the compound formed by the dehydration of L-arabonate agree well with those described for the keto acid formed from D-arabonate. It seems probable, therefore, that these two compounds are stereoisomers of each other.

SUMMARY

A new intermediate, L-2-keto-4,5-dihydroxyvaleric acid, has been identified in the oxidation of L-arabinose to α-ketoglutaric acid by *Pseudomonas saccharophila*. It is formed by a nonoxidative process from L-arabonate and is isolated by elution from a Dowex 1 column. The intermediate has been characterized by chemical degradative procedures and by reducing it to 2,4,5-trihydroxyvaleric acid.

In the presence of diphosphopyridine nucleotide the intermediate is quantitatively oxidized to α-ketoglutaric acid at a much greater rate than is L-arabonate. It is suggested that at least two steps are required for this conversion, an oxidation being the

first step. The enzyme system seems to exhibit a specificity for the L-configuration since in this system only 50 per cent of the DL-2-keto-4,5-dihydroxyvaleric acid are oxidized, and D-arabonate not at all.

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REFERENCES

1. Weimberg, R., and Doudoroff, M., *J. Biol. Chem.*, **217**, 607 (1955).
2. Palleroni, N. J., and Doudoroff, M., *J. Bacteriol.*, **74**, 180 (1957).
3. Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949).
4. MacFadyen, D. A., *J. Biol. Chem.*, **158**, 107 (1945).
5. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
6. Lugg, J. W. H., and Overell, B. T., *Nature*, **160**, 87 (1947).
7. Umbarger, H. E., and Magasanik, B., *J. Am. Chem. Soc.*, **74**, 4253 (1952).
8. Abdel-Akher, M., and Smith, F., *J. Am. Chem. Soc.*, **73**, 5859 (1951).
9. Cavallini, D., Frontali, N., and Toschi, G., *Nature*, **164**, 792 (1949).
10. Hockenhull, D. J. D., and Foodgate, G. D., *Biochem. J.*, **52**, 38 (1952).
11. Moore, S., and Link, K. P., *J. Biol. Chem.*, **133**, 293 (1940).
12. Nef, J. V., *Ann. Chem. Liebigs*, **376**, 1 (1910).
13. Glattfeld, J. W. E., Leavell, G., Spieth, G. E., and Hutton, D., *J. Am. Chem. Soc.*, **53**, 3164 (1931).
14. Glattfeld, J. W. E., and Rietz, E., *J. Am. Chem. Soc.*, **62**, 974 (1940).
15. MacGee, J., and Doudoroff, M., *J. Biol. Chem.*, **196**, 853 (1952).
16. Lanning, M. C., and Cohen, S. S., *J. Biol. Chem.*, **189**, 109 (1951).
17. Palleroni, N. J., and Doudoroff, M., *J. Biol. Chem.*, **223**, 499 (1956).
18. Entner, N., and Doudoroff, M., *J. Biol. Chem.*, **196**, 853 (1952).
19. De Ley, J., and Doudoroff, M., *J. Biol. Chem.*, **227**, 745 (1957).

The Catalytic Decarboxylation of Pyruvate by Thiamine*

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Thiamine diphosphate is essential to the action of enzymes catalyzing the decarboxylation of α-keto acids, the formation of α-keto alcohols and related reactions. The thiamine diphosphate molecule while combined with the enzyme is probably intimately involved in the catalytic process. Ugai *et al.* (1) and Mizuhara *et al.* (2) and Mizuhara and Oono (3) have shown that thiamine itself catalyzes these same reactions slowly in the absence of any enzymes. Because study of these nonenzymic reactions should assist in understanding the biological function of thiamine, we have investigated further the decarboxylation of pyruvate catalyzed by thiamine under conditions similar to those of Mizuhara. The stoichiometry and kinetics of the reaction, identity of products and effects of pH, metal ions, and temperature have been considered. Similar work has been reported briefly by Koffler and Krampitz (4).

EXPERIMENTAL

Chemicals and Reagents—Sodium pyruvate from Nutritional Biochemicals Corporation was recrystallized from ethanol (5). Merck thiamine chloride hydrochloride was used as received. Stock solutions (0.5 M) of these two compounds were prepared, stored in a refrigerator, and used for up to 1 month. Crystalline acetoin dimer was prepared from commercial acetoin which had been redistilled under vacuum (6). Acetolactate solutions were obtained by hydrolysis of ethyl α-methyl-α-acetoxyacetoacetate prepared by the method of Krampitz (6). Buffers were prepared from recrystallized borax or sodium pyrophosphate and HCl or recrystallized triethanolamine hydrochloride and NaOH.

Procedure—Reactions were conducted in Thunberg tubes which had been evacuated to about 15 mm. pressure with a water pump for 5 to 15 minutes, in Warburg vessels under nitrogen, or under nitrogen in special reaction vessels. The latter permitted sampling at various time intervals by means of a hypodermic syringe needle inserted through a rubber cap. In the majority of cases the main chamber contained the buffer, sodium pyruvate, water, and sufficient NaOH to bring the thiamine added to the desired pH. In the Thunberg cap or reaction vessel side arm was placed 0.1 to 0.2 ml. of thiamine hydrochloride solution.

It was found that pyruvate was surprisingly labile when exposed to moderate excesses of base. Triethanolamine buffers (pK 7.9) in contrast to borate buffers (pK 9.2) had insufficient capacity to prevent the pH of the pyruvate-buffer-NaOH solution from rising to about 12 before the thiamine hydrochloride was tipped in. As much as 30 per cent of the pyruvate was destroyed during the 30 minutes required for preparation, evacuation, and preincubation of the samples. This difficulty was avoided in the Warburg manometer runs by using neutralized thiamine in the side arm in place of thiamine hydrochloride. In the Thunberg tube runs it was found easier to put the pyruvate in the cap and the remainder of the reaction mixture including the thiamine in the tube. This modified procedure was used in obtaining the data on pH dependence in Fig. 3. Controls containing thiamine and buffer alone and pyruvate and buffer were always used. The total volume after mixing was usually 2.5 ml. For the longer incubations in Warburg flasks all volumes were cut by one-half. For measuring the kinetics of acetoin and acetolactate formation larger volumes were incubated in special reaction vessels.

When acetaldehyde was required in a reaction mixture, the evacuated Thunberg tubes were filled with nitrogen to a pressure slightly in excess of 1 atmosphere. After several minutes in the water bath, excess pressure was released by opening the tube momentarily. The tube was then again opened momentarily and the acetaldehyde injected by means of a syringe.

pH values were measured, usually on each reaction mixture, at 25° with a Beckman model G pH meter. In most cases the desired pH values held to within ±0.1 unit during the reactions. The pK_a of the thiazolium ring-opening reaction was measured as the pH of a solution of pure recrystallized thiamine chloride hydrochloride to which had been added exactly two equivalents of NaOH.

Analytical Methods—Acetoin was measured by the procedure of Westerfeld (7) in which it reacts with creatine, α-naphthol, and oxygen in a basic medium to give a red color. The optical density was measured at 525 mμ with a Beckman spectrophotometer. The molar absorbancy index, based on acetoin concentration, was 2.01×10^4. Standards were prepared from crystalline acetoin dimer. The chemistry of this color reaction is unknown, but we have performed the following tests to establish its reliability. Good agreement was observed when an acetoin-containing mixture produced by the incubation of thiamine with pyruvate was analyzed by both the Westerfeld method and the procedure of Neuberg and Strauss (8). In the latter, acetoin is oxidized to biacetyl, distilled, and converted to the bis-2,4-dinitrophenylhydrazone which is measured colorimetrically. Furthermore the 2,4-dinitrophenylhydrazones prepared in this way from (*a*) authentic acetoin, (*b*) a thiamine-pyruvate reaction mixture, and (*c*) a mixture of (*a*) and (*b*), all had the same melting point of 315–318° with decomposition.

The effects on the acetoin color test of all the components used

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in our reaction mixtures in the maximal amounts ever present have been studied. The color developed from 0.1 μmole of acetoin was unaffected by borate and sodium pyruvate. Thiamine and other thiazolium salts diminish the color yield, more so if no pyruvate is present. When 20 μmoles of thiamine and 200 μmoles of pyruvate were present only 77 per cent of the expected color formed. This interference was not significant in the present experiments where the thiamine content in samples for analysis seldom exceeded 4 μmoles and the color yield was at least 96 per cent of maximum. The thiol form of thiamine (form III, see diagram in "Results and Discussion") is presumed to interfere with the color reaction as do other thiols (9).

When acetaldehyde is present the color is atypical. Color development is inhibited and does not become maximal until after 24 hours. This interference was avoided by removal of the excess acetaldehyde by aeration from aliquots of reaction mixtures (usually 0.5 ml.) over a period of 3 hours at room temperature. Air was passed into the samples using 5-inch stainless steel syringe needles. To stop acetolactate formation during the aeration, the pH of the solutions was lowered to about 6 by addition of HCl or of phosphate buffers.

A small amount of color is developed by thiamine-containing blanks upon long incubation. Thiamine decomposition is known to lead to an α-keto mercaptan (10, 11) which could possibly give the color test. The error in our measurements from this cause is insignificant unless pyruvate in some way promotes the breakdown of thiamine to color-yielding products.

Acetolactate gives little if any color in the acetoin test. However, it can readily be decarboxylated to acetoin by treatment with acid (6). We find that addition of sufficient hydrochloric acid to lower the pH to 2 or less, followed by 1 hour of incubation at 40°, will quantitatively decarboxylate acetolactate. Analysis for acetoin then gives the sum of the acetoin and acetolactate originally present.

Acetaldehyde was measured by the direct aeration procedure described by Neidig and Hess (12). The color produced was measured at 575 mμ with the use of a Beckman model B spectrophotometer. Standard acetaldehyde solutions were aerated with each set of samples. These solutions could be standardized conveniently by comparing their color yields (without aeration) with that of a standard paralydehyde solution prepared according to Stotz (13).

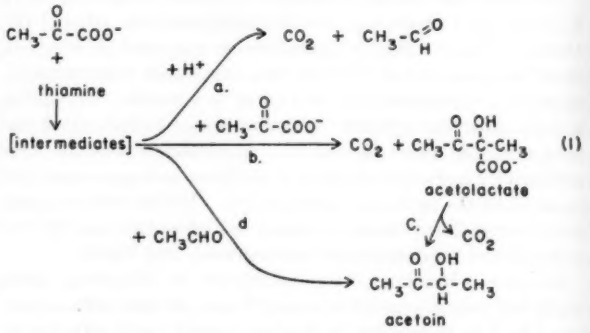
Pyruvate was determined by a modification (14) of the method of Friedemann and Haugen.

Thiamine was measured by conversion to the transient yellow form (15) according to the following procedure. The sample was diluted and slightly acidified to convert all thiamine present to the thiazolium form. Into a 1-cm. spectrophotometer cell were placed 3 ml. of the diluted sample and the spectrophotometer was set at 100 per cent transmission. At zero time, 0.1 ml. of 2.6 N sodium hydroxide was added with the Boyer-Segal addermixer (16). Readings were taken at 349 mμ every 5 seconds for 60 seconds. The plot of log absorbancy against time was linear and permitted extrapolation to obtain the zero time absorbancy. The test appears to be highly specific for the intact thiamine molecule.

Carbon dioxide evolution was determined in the Warburg apparatus using flasks with two side arms. The first side arm contained the thiamine solution, which was tipped in at zero time; the second, 0.1 ml. of 5 N sulfuric acid to stop the reaction and release the carbon dioxide. Excessive carbon dioxide absorption from the air during sample preparation was avoided by working rapidly and by neutralizing the thiamine solution placed in the side arm so that a smaller excess of base would be required in the main chamber before mixing. This also prevented the previously described destruction of pyruvate in triethanolamine buffers. No carbon dioxide was produced when pyruvate and buffer were incubated alone. A very slight apparent carbon dioxide production occurred as thiamine blanks were incubated, amounting to less than 0.3 mM (0.3 mmole of carbon dioxide produced per liter of reaction mixture) in 3 hours. Since this amount is barely significant, no correction was made. Reaction mixtures into which the acid was tipped at zero time evolved about 1.2 mM carbon dioxide. All data were corrected through the use of such controls with each experiment. Samples were run in triplicate and the results averaged. Carbon dioxide evolution from carbonate was complete 10 minutes after the acid was tipped but when acetolactate was present a full 50 minutes was needed.

RESULTS AND DISCUSSION

When sodium pyruvate is incubated with thiamine in slightly alkaline solution in the absence of air, the pyruvate is slowly decarboxylated. Several products may arise. Presumably thiamine and pyruvate initially react to form some type of intermediate compound or compounds. Either before or after decarboxylation such an intermediate may react with a proton (Equation 1*a*) to produce acetaldehyde and carbon dioxide, or with the carbonyl group of a second molecule of pyruvate to yield α-acetolactate (Equation 1*b*).



(1)

This latter β-keto acid may be readily decarboxylated (Equation 1*c*) to acetoin. Acetaldehyde, if present, may also react in step *d* of Equation 1 to give acetoin directly.

Under our test conditions acetaldehyde is not produced to a significant extent, but the reaction proceeds via steps *b* and *c* to give acetolactate and acetoin as the major products.

Acetoin and Carbon Dioxide Production—Fig. 1 shows the formation of carbon dioxide (*Curve A*) and of acetoin (*Curves B* and *C*) when 0.2 M pyruvate was incubated with 0.02 M thiamine in triethanolamine or borate buffer, pH 8.9 to 9.0, 40°. The acetoin concentrations have been multiplied by two because 2 molecules of pyruvate must be decarboxylated to yield 1 of acetoin. The acetoin after acid treatment (*Curve D*) represents the sum of acetoin plus acetolactate plus any other intermediates which might accumulate and yield acetoin upon acidification.

The rate of acetoin production eventually falls off. In one experiment in 40 hours 22 mM acetoin was produced in borate buffer (without acid treatment). This corresponds to a loss of

22 per cent of the pyruvate present. However, direct measurement showed that over 90 per cent of the pyruvate had been destroyed. Even in 4 hours 20 per cent of the pyruvate was destroyed, whereas carbon dioxide evolution accounted for only 4.7 per cent of this. Evidently some nondecarboxylative side reactions occur. Pyruvate undergoes a variety of base-catalyzed condensations (17) and it is possible that thiamine promotes some of these. Pyruvate destruction in controls containing no thiamine was only about half as great as in the presence of thiamine. Thiamine was also destroyed in the same reaction mixtures to the extent of approximately 5 per cent in 4 hours and 24 per cent in 40 hours at 40°.

Acetoin Production from Acetaldehyde—Thiamine also catalyzes the direct acyloin condensation of 2 molecules of acetaldehyde to acetoin. However, acetaldehyde reacts much more slowly than pyruvate. When approximately 0.2 M acetaldehyde was incubated with 0.02 M thiamine at pH 9.2 in 0.08 M pyrophosphate buffer for 2 hours at 40° only 0.1 mM acetoin was formed. This is only about 6 per cent of that formed from 0.2 M pyruvate under comparable conditions.

Thiamine as Catalyst—Even in 40 hours each molecule of thiamine present has catalyzed the formation of only a little over 1 molecule of acetoin on the average. To clearly establish that thiamine is acting *catalytically*, we have devised conditions (0.01 M thiamine, 0.5 M pyruvate, borate buffer, pH 8.7, 50°) under which a yield of 3 moles of acetoin per mole of thiamine is observed in 22 hours.

Kinetics and Evidence for Unstable Intermediate—The evolution of carbon dioxide exceeds that of acetoin plus acetolactate (Fig. 1). Carbon dioxide production begins immediately and is readily measurable after 15 minutes. However, in 15 minutes no acetoin or acetolactate is yet detectable and there is a lag of over an hour before the rate of acetoin formation becomes maximum. We believe that this characteristic lag together with the rapid initial carbon dioxide production indicates the formation of one or more unstable intermediates.

The kinetics of the reaction can be described approximately by assuming two consecutive first order reactions. The equations for this simple case (18) can be fitted to the data remarkably well. On this basis, from *Curve D* (Fig. 1) a maximal intermediate concentration of 0.75 mM is estimated. However, several complications prevent an exact mathematical description: (*a*) The "steady-state" rate of carbon dioxide formation is about 17 per cent greater than that of acetoin (× 2), probably because some of the decarboxylated pyruvate is converted to side products. (*b*) The amount of carbon dioxide evolved in the first 15 minutes of reaction is too high. The dashed portion of *Curve A* (Fig. 1) indicates the expected shape of the carbon dioxide curve for two consecutive first order reactions. We have been unable to find the cause of this anomaly. (*c*) The previously mentioned destruction of pyruvate and thiamine causes the reaction rate to fall prematurely.

Further evidence for the presence of a reactive intermediate was obtained as follows. Pyruvate (0.2 M) and thiamine (0.04 M) were incubated in 0.08 M pyrophosphate buffer, pH 9.2, 40° for 30 minutes. Enough acetaldehyde to give a concentration of 0.2 M was then added to one such sample (C) while to another (A) was added a comparable amount of water. Sample A was then placed in an ice bath to hinder further reactions. Sample C was incubated another 30 minutes and acetoin was determined *without* decarboxylation. Still a third sample (B) containing

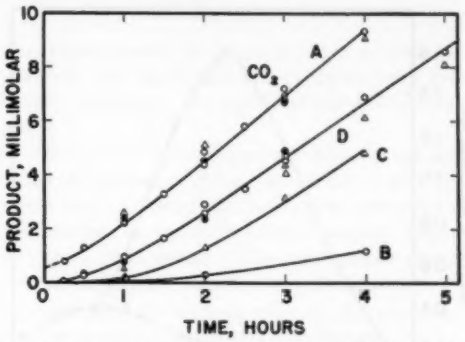


FIG. 1. Acetoin and carbon dioxide produced by incubation of pyruvate with thiamine at pH 8.9. 40°. ○, ●, triethanolamine-buffered; △, borate-buffered. ○, △, 0.2 M pyruvate + 0.02 M thiamine; ●, 0.4 M pyruvate + 0.01 M thiamine. *Curve A*, carbon dioxide evolved after acid treatment. *Curves B* and *C*, acetoin (× 2) without acid treatment. *Curve D*, acetoin (× 2) after acid treatment to decarboxylate acetolactate.

pyruvate, thiamine, and acetaldehyde, all present initially, was incubated for a total of 30 minutes. The amount of acetoin (solid bar, Fig. 2) formed in sample C far exceeded that in samples A and B combined. Apparently an intermediate accumulated during the first 30 minutes in sample C. When acetaldehyde was added some of the intermediate reacted with it to give acetoin directly. In sample A the small amount of acetoin formed came indirectly through decarboxylation of acetolactate, whereas in sample B a relatively small amount of acetoin formed because the intermediate had to accumulate before condensation with acetaldehyde could take place.

The relative efficiencies of pyruvate and acetaldehyde as "acceptors" for the intermediate can be judged roughly by the amounts of acetolactate (0.05 mM) and of acetoin (0.80 mM) formed during the last 30 minutes of incubation of sample C. The acetolactate value was calculated by subtracting the sum of solid bar C and lined bar A from lined bar C in Fig. 2. The acetoin value was taken as just equal to solid bar C since little

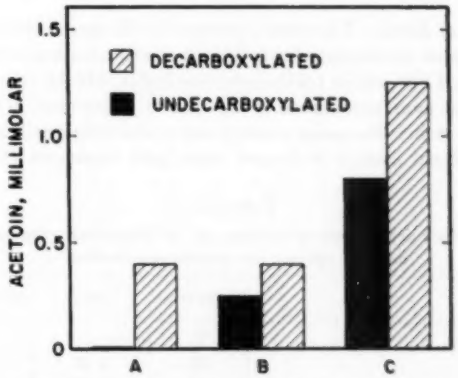


FIG. 2. Reaction of acetaldehyde with accumulated intermediate. All samples contained 0.2 M pyruvate, 0.04 M thiamine, and pyrophosphate buffer, pH 9.2, and were incubated for 30 or 60 minutes at 40°. Acetoin was measured directly as well as after acid treatment to decarboxylate acetolactate. A, 30 minutes, with no acetaldehyde; B, 30 minutes, with 0.2 M acetaldehyde; C, 60 minutes, with acetaldehyde (to give 0.2 M) added at 30 minutes.

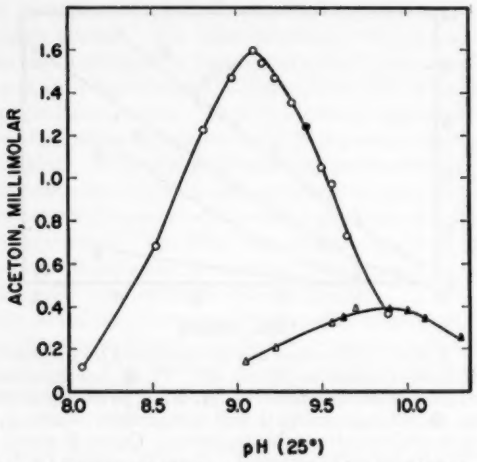


FIG. 3. pH dependence of acetoin formation catalyzed by thiamine and by 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride. Pyruvate (0.2 M) and catalyst (0.02 M) were incubated at 40° for 2 hours and the acetoin measured after acid treatment. ○, ●, thiamine; △, ▲, 3-benzyl analog; ○, triethanolamine buffers; △, pyrophosphate buffers; ●, ▲, no buffer added.

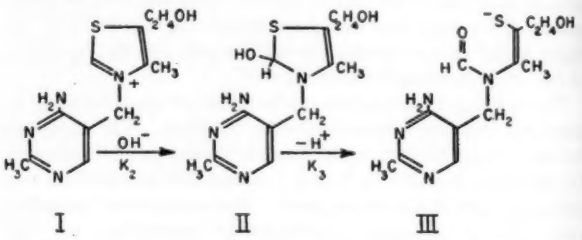
acetoin *per se* is produced in 1 hour without acid treatment and acetaldehyde alone as a substrate gives very little acetoin. These values show that acetaldehyde is much superior to pyruvate as an "acceptor." A strikingly analogous behavior has been demonstrated for pyruvic acid oxidase preparations from pigeon breast muscle by Juni and Heym (19).

The kinetically detectable intermediate is probably a decarboxylated compound of thiamine with pyruvate, *i.e.* a thiamine-acetaldehyde compound. It could equally well be a thiamine-acetolactate compound in equilibrium with the thiamine-acetaldehyde compound. We felt that it might be possible to break the intermediate down to acetaldehyde by treating with acid. However, repeated attempts to obtain acetaldehyde by either acid or base treatment have failed. At most, less than 1 to 2 per cent of the intermediate has been obtained as acetaldehyde.

Effect of Borate—The rates of carbon dioxide production and of acetoin plus acetolactate formation are nearly identical in borate buffer, pH 8.9, and in triethanolamine buffer, pH 9.0 (the lag is somewhat more pronounced in the former). However, in borate buffer acetoin is the major product and a relatively small amount of additional acetoin is formed upon acid treatment (Fig. 1) whereas in triethanolamine buffer or unbuffered solution acetolactate is the major product. Boron, 0.05 gm. atoms per liter, suffices to give the maximal yield of acetoin. The following experiments with freshly prepared acetolactate show that this compound is quickly decarboxylated in borate buffer at pH 8.8.

Acetolactate solutions when prepared by careful addition of base at 0° under nitrogen to ethyl α-methyl-α-acetoxyacetoacetate gave a color yield in the acetoin test of only 4 to 6 per cent of that given by the same number of moles of acetoin. Either 4 to 6 per cent of the compound is decarboxylated during hydrolysis or acetolactate actually gives a small amount of color in the test. When such an acetolactate solution is decarboxylated by incubation at 40° at pH 2, 93 to 99 per cent of the theoretical acetoin is produced. When the acetolactate is incubated at pH 8.8 to 9, a slow production of acetoin is observed in triethanolamine buffer and a much more rapid one in borate buffer. In the latter case the reaction of 1 mM acetolactate is nearly complete in 1 hour. Surprisingly, the final yield of acetoin from borate decarboxylation is only 49 to 56 per cent of theory. If, after prolonged (2 hour) incubation in borate buffer, acid is added, 90 to 92 per cent of the theoretical acetoin is recovered. Apparently in the borate buffer some of the acetolactate is converted to a form resistant to decarboxylation by borate but which can be decarboxylated by acid.

Effect of Metal Salts—Thiamine diphosphate-requiring enzymes also require divalent metal ions for activity. However, no effects on the rate of acetoin production in the model system could be detected with Ca, Mg, Co, Fe^{+++}, Mn, Al, or Cd salts. A slight stimulation of acetoin production in triethanolamine buffer was caused by Pb^{++} and Sn^{++} salts. It was later shown that these salts, like borate, act to decarboxylate catalytically any acetolactate formed.



pH and Temperature Dependence—Fig. 3 shows the variation with pH of the amount of acetoin plus acetolactate produced in 2 hours at 40° in triethanolamine buffer. The rate was maximal at pH 8.9 (40°) and was insignificant below pH 7.5. The pH values were measured at 25° and were found to be about 0.2 units higher than when measured at 40°. The pH optimum is very close to the pK (average of pK_2 and pK_3), for the conversion of the thiazolium ion form of thiamine I to the pseudo-base and thiol forms II and III.

We have measured the average pK as a function of temperature (see Table I) and find an almost linear dependence between 15° and 40°. Watanabe and Asahi (20) have published a somewhat different dependence. The value at 40° is 8.96. Mizuhara *et al.* (2) found from data involving the addition of one, two or three equivalents of base per equivalent of thiamine[1] that the greatest acetoin yield came from the two to one ratio sample.

TABLE I

Temperature dependence of average pK of thiazolium ring-opening and pH optima for acetoin production

	Temperature	pK_{av}	pH optima
	°C.		
Thiamine	15	9.43	
	20	9.33	
	25	9.23	
	30	9.15	
	35	9.05	
	40	8.96	8.9
3-Benzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride	40	9.63	9.7

[1] The pH values reported for these samples are curiously low, being 5.8, 8.4, and 9.2 respectively.

From these data, Mizuhara concluded that the pseudo-base II is the catalytically active form of thiamine. Its concentration will be maximal at the pK_{ave}.

However, the observed pH dependence does not rule out other forms of thiamine as the catalytically effective ones. For example, if a thiazolium dipolar-ion is the catalytically active form (see "Discussion" in reference 21) as proposed by Breslow (22), its concentration will also be maximal at the pK_{ave}.

Fig. 3 shows also the pH dependence of the same reaction catalyzed by a 3-benzyl analogue of thiamine. The average pK of this compound is 9.6 at 40° and the maximal rate is again very close to this pK. This compound is discussed further in an accompanying paper (21).

Increasing the temperature increases the reaction rate but above 50° the advantage is small because of the more rapid destruction of thiamine. Therefore we have used either 40 or 50° in our experiments.

SUMMARY

Thiamine catalyzes the decarboxylation of pyruvate to acetoin, α-acetolactate, and small amounts of unidentified products in slightly basic solutions. No significant amount of acetaldehyde is formed.

Carbon dioxide evolution begins immediately but acetolactate and acetoin appearance exhibit a characteristic lag. The kinetics are approximately those of two consecutive first order processes.

An intermediate accumulates which is capable of reacting with acetaldehyde to yield acetoin. The intermediate cannot be converted to acetaldehyde by acid or base treatment.

Most metal salts are without effect on the reaction. Borate catalyzes the decarboxylation of α-acetolactate.

The pH optimum is approximately 8.9 at 40°.

The temperature dependence of the equilibrium constant for the thiazolium ring-opening reaction has been measured.

REFERENCES

1. Ugai, T., Tanaka, S., and Dokawa, S., *Yakugaku Zasshi*, **63**, 296 (1943).
2. Mizuhara, S., Tamura, R., and Arata, H., *Proc. Japan Acad.*, **27**, 302 (1951).
3. Mizuhara, S., and Oono, K., *Proc. Japan Acad.*, **27**, 705 (1951).
4. Koffler, H., and Krampitz, L. O., *Bacteriol. Proc.*, 113 (1955).
5. Price, V. E., and Levintow, L., *Biochem. Preparations*, **2**, 23 (1952).
6. Krampitz, L. O., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. III*, Academic Press, Inc. New York, 1957, p. 277.
7. Westerfeld, W. W. *J. Biol. Chem.*, **161**, 495 (1945).
8. Neuberg, C., and Strauss, E., *Arch. Biochem.*, **7**, 211 (1945).
9. Ennor, A. H., and Stocken, L. A., *Biochem. J.*, **42**, 557 (1948).
10. Matsukawa, T., Iwatsu, T., and Yurugi, S., *Yakugaku Zasshi*, **71**, 369 (1951).
11. Watanabe, A., and Asahi, Y., *Yakugaku Zasshi*, **77**, 153 (1957).
12. Neidig, B. A., and Hess, W. C., *Anal. Chem.*, **24**, 1627 (1952).
13. Stotz, E., *J. Biol. Chem.*, **148**, 585 (1943).
14. Metzler, D. E., and Snell, E. E., *J. Am. Chem. Soc.*, **74**, 979 (1952).
15. Maier, G. D., and Metzler, D. E., *J. Am. Chem. Soc.*, **79**, 4386, 6583 (1957).
16. Boyer, P. D., and Segal, H. L., in W. D. McElroy and B. Glass (Editors), *The mechanism of enzyme action*, Johns Hopkins Press, Baltimore, 1954, p. 523.
17. Waldmann, E., Prey, V., and Jelinek, F., *Monatsch. Chem.*, **85**, 872 (1954).
18. Moelwyn-Hughes, E. A., *Physical chemistry*, Cambridge University Press, London, 1951, p. 639.
19. Juni, E., and Heym, G. A., *J. Biol. Chem.*, **218**, 365 (1956).
20. Watanabe, A., and Asahi, Y., *Yakugaku Zasshi*, **75**, 1046 (1955).
21. Yount, R. G., and Metzler, D. E., *J. Biol. Chem.*, **234**, 738 (1959).
22. Breslow R., *Chem. & Ind.*, London, 893 (1957).

Decarboxylation of Pyruvate by Thiamine Analogues*

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Thiamine catalyzes the nonenzymic decarboxylation of pyruvate to α-acetolactate and acetoin (1–4) in mildly basic aqueous solution. In order to study the mechanism of this reaction we have tested a number of thiamine analogues as catalysts. Breslow (5) has reported some very similar results which we confirm.

EXPERIMENTAL

Sources of Compounds Tested—Thiamine hydrochloride (Merck, U.S.P. grade), *O*-acetylthiamine hydrochloride and oxythiamine dihydrochloride (California Foundation for Biochemical Research), pyrithiamine hydrobromide and 4-amino-5-aminomethyl-2-methyl pyrimidine dihydrochloride (Nutritional Biochemicals Corporation) were all used without further purification. For determination of pK_a values, the 4-amino-5-aminomethyl-2-methyl pyrimidine dihydrochloride was recrystallized from ethanol and ether until it gave the theoretical end point when titrated with base.

5-(2-Hydroxyethyl)-4-methyl thiazole was redistilled from pooled commercial products of Merck[1] and du Pont. The boiling point was 108°, 0.8 mm. Infrared inspection of the redistilled product showed the loss of a strong carbonyl peak which was originally present. The purified product was stored in a desiccator at −20° to hinder oxidation.

Salts of 5-(2-Hydroxyethyl)-4-methyl Thiazole—The method of Clarke (6) was employed to prepare the 3-methyl and 3-benzyl (7) and the 3-*o*-, *m*-, and *p*-nitrobenzyl (8) salts.

The 3-α-methylbenzyl salt was prepared by dissolving 0.04 mole each of α-bromoethyl benzene (Eastman, redistilled 89°, 15 mm.) and 5-(2-hydroxyethyl)-4-methyl thiazole in 4 ml. of thiophene-free anhydrous benzene in a stoppered 18 × 150-mm. test tube. The solution was heated overnight at 55°. The α-methylbenzyl salt separates as a light yellow syrup. Attempts to crystallize this compound from a number of solvent systems were all unsuccessful. During crystallization attempts in hot absolute ethanol the salt was partially solvolyzed to the pyrimidylmethyl ethyl ether and 5-(2-hydroxyethyl)-4-methyl thiazole hydrobromide. The absorption spectra in both acid and base and the titration behavior very closely paralleled those of the 3-benzyl salt. These properties plus the method of preparation leave little doubt as to the character of the product.

The 3-cyanomethyl salt was prepared in a similar manner from chloroacetonitrile (Eastman white label, redistilled 24°, 27 mm.) and 5-(2-hydroxyethyl)-4-methyl thiazole. The solution was heated at 80° for 3 days, the benzene supernatant was discarded, and the red resinous layer which remained was triturated twice with anhydrous ethyl ether to remove as much as possible of the starting materials and benzene solvent. The gummy mass remaining was dissolved in 25 ml. of absolute ethanol and was treated twice with about 0.5 gm. of charcoal (Darco). Anhydrous ethyl ether was added to the point of incipient turbidity and the solution cooled overnight at 2°C. The dense bright yellow crystals were collected and washed with cold ethanol and ethyl ether (50:50). The yield after recrystallization was 3.2 gm., m.p. 132–133°. Repeated recrystallization, charcoal treatment, and chromatography on Dowex 50-X4 exchange resin with 0.2 N HCl as the developing solvent failed to remove the yellow color. However, the ultraviolet absorption spectrum was typical of a thiazolium compound in both acid and basic solution and exhibited negligible absorption above 300 mμ.

$C_8H_{11}ClN_2OS$

Calculated:	C 44.54, H 5.14, Cl 16.22, N 12.99
Found:	C 43.93, H 5.07, Cl 15.9, N 12.81

Surprisingly, the infrared spectrum does not contain a cyanide peak in the region of 4.5μ.

O-Heterothiamine (3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)-4-methyloxazolium chloride) was generously give to us by Dr. John F. Codington of the Sloan-Kettering Institute for Cancer Research.

3-[(4-Amino-2-methyl-5-pyrimidinyl)-methyl]-4-methyl Oxazolium Bromide—4-Methyl oxazole was prepared by the method of Cornforth and Cornforth (9). 4-Methyl oxazole (0.02 mole) was dissolved in 3 ml. of freshly distilled *n*-butanol and heated to 95–100°. 4-Amino-5-bromomethyl-2-methyl pyrimidine dihydrobromide (0.067 mole, Merck, technical grade) was added in small portions and mixed well. The test tube was tightly stoppered and heated for 30 minutes more at 125° with shaking at 4 to 5 minute intervals. The solution was cooled and the crystals which formed were collected and washed with butanol and then ether. The crystals were dissolved in a minimal amount of water, 95 per cent ethanol was added to the point of incipient turbidity, and the solution was cooled to 2° for 2 days. The resultant crystals were collected, washed, and recrystallized to yield 0.23 gm. of small, light tan crystals which melted with decomposition at 235°.

$C_{10}H_{14}Br_2N_4O$

Calculated:	C 32.88, H 3.86, N 15.3, Br 43.66
Found:	C 32.84, H 4.04, N 15.3, Br 43.7

Dihydrothiamine and tetrahydrothiamine were prepared by the borohydride reductions of thiamine described by Bonvicino and Hennessy (10).

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† Procter and Gamble Fellow; Summer 1957.

[1] Donated by Dr. Karl Folkers of Merck and Company, Inc.

3-(3-Aminopropyl)-4-methyl thiazolium chloride and 3-(4-aminobutyl)-4-methyl thiazolium chloride were from the collection of the late R. R. Sealock and were prepared by Sarver (11) using the method of Clarke (6).

Testing Procedure—The compounds were tested by incubation with sodium pyruvate in evacuated Thunberg tubes at 50° in borate or pyrophosphate buffers as previously described (4). Acetoin was then measured, usually without acid treatment (*i.e.* acetolactate present was not decarboxylated).

Accurate estimates of relative catalytic activity are especially difficult with the poorer catalysts. Because of the pronounced lag in acetoin production the amounts of acetoin measured are very small. Fortunately the acetoin color test is very sensitive though its accuracy is diminished by the presence of large amounts of thiazolium compounds (4). More vexing is the problem of high blanks. Sodium pyruvate and thiamine itself both cause a very small amount of color. The color from thiamine may arise by hydrolytic breakdown to the ketol $CH_3COCHOH-CH_2CH_2OH$ which would probably give the color test. The related thiol is a known degradation product of thiamine (12) and might also yield some color. The methyl thiazolium salt (3,4-dimethyl-5-(2-hydroxethyl) thiazolium chloride) appears to break down in the same way. Since it is a much less active catalyst than thiamine, the blanks become a serious problem. At pH 9 or below these blanks were negligible, but above 9 they increased rapidly.

If the sulfur of thiamine is replaced by oxygen, the resulting oxazolium salt (*O*-heterothiamine) decomposes gradually to a color-yielding material. The formation of the previously mentioned ketol should occur readily in this compound. An accurate estimate of its catalytic activity was therefore impossible. However, no such interference was observed with the 4-methyl oxazolium analogue of thiamine.

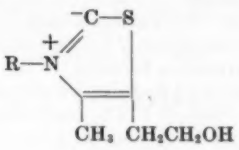
RESULTS AND DISCUSSION

One of the better catalysts studied is the benzyl analogue of thiamine, 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride (VIII, Table I). As with thiamine (I) catalysis, a characteristic lag in acetoin production is observed. Because of this lag, the relative catalytic activities of two compounds cannot be compared by simply measuring the amounts of acetoin produced in a given length of time. Thus in 1 hour in borate buffer thiamine catalysis produces 7 times as much acetoin as that of the benzyl analogue at the same pH; in 3 hours it is only 3.5 times as much. It is more proper to compare the lengths of time required for the production of a certain concentration of acetoin under specified conditions. Thus with the benzyl analogue the time required to produce any amount of acetoin in pH 8.8 borate buffer at 50° is always 2.6 times that required with thiamine as catalyst. If the amounts of acetoin produced by action of the benzyl analogue are plotted against heating time divided by 2.6, the points fall on the curve of acetoin *versus* time for thiamine catalysis (Fig. 1). Thus we conclude that the benzyl analogue is 1/2.6 times as active as thiamine at this pH.

Similarly we can conclude that the rate of production of acetoin by thiamine at 50° is just 3 times as fast as at 40° (Fig. 1). Apparently temperature and catalyst structure influence the rate in similar ways.

The activities of a series of other catalysts have also been measured. The activities relative to that of thiamine are summarized in Table I.

The results show that in nonenzymic catalysis of acetoin formation the thiazolium group of thiamine must be the site of interaction with pyruvate. The 3-methyl thiazolium analogue (X) is slightly active and the 3-benzyl analogue (VIII) is 38 per cent as active as thiamine. On the other hand, pyrithiamine (III) is completely inactive despite the fact that the aminopyrimidine group and the quarternary nitrogen are present as in thiamine. These results are in agreement with those of Breslow (5, 13). Another possible site of interaction with thiamine, the methylene bridge, has been ruled out by work of Fry *et al.* (14) and Breslow (13). Breslow has furthermore provided convincing evidence in favor of the thiazolium dipolar ion XVIII



XVIII

as an intermediate in the reaction with pyruvate (13, 15). Some further support is lent to Breslow's suggestion by the inactivity of 3-benzyl-, phenyl-, and allyl-2,4-dimethyl thiazolium salts (Downes and Sykes, 16) and of 3-benzyl-2,4-dimethyl thiazolium bromide in catalysis of the furoin condensation (Ugai *et al.*, 17). We have previously reported (18) that the 2,4-dimethyl thiazolium analogue of thiamine (V) is also inactive. However, we have not obtained this compound in a satisfactory state of purity, and this result must be regarded as tentative.

According to the Breslow mechanism the α-methylbenzyl analogue of thiamine (IX) might be anticipated to be an active catalyst. There may be two reasons for its inactivity. First, the addition of the methyl group on the methylene bridge may facilitate the solvolysis of the compound at the quaternary nitrogen so that the molecule splits before it has a chance to condense with pyruvate. Secondly, the additional methyl group may sterically hinder the attack of the dipolar ion on pyruvate, although scale models seem to indicate that this hindrance should not be great.

In addition, the series of *o*-, *m*-, and *p*-nitrobenzyl analogues

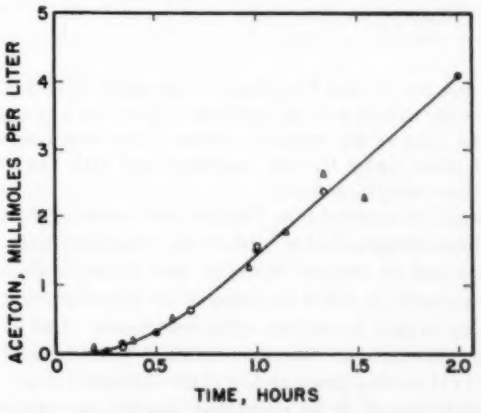
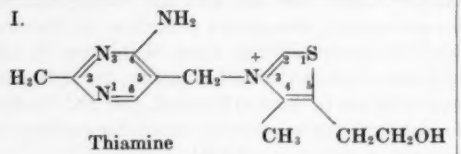


FIG. 1. Acetoin production *versus* time for two catalysts. Pyruvate (0.2 M) plus catalyst (0.02 M) incubated at pH 8.8 to 9, borate buffer. ●, Thiamine as catalyst, 50°; ○, thiamine 40°, time divided by 3.0; △, 3-benzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride, 50°, time divided by 2.6.

TABLE I

Relative activities in catalysis of pyruvate decarboxylation to acetoin

Catalyst	Relative activity pH 8.7–9.0
I.  Thiamine	100
II. *O*-Acetylthiamine	100
III. Pyrithiamine, 1-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-3-(2-hydroxyethyl)-2-methyl pyridinium bromide	0
IV. Oxythiamine, 3-[(4-hydroxy-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride	Trace (20 at pH 10.2)
V. 3-[(4-Amino-2-methyl-5-pyrimidinyl)-methyl]-2,4-dimethyl thiazolium bromide	0?
VI. *O*-Heterothiamine, 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)-4-methyl oxazolium chloride	0
VII. 3-[(4-Amino-2-methyl-5-pyrimidinyl)-methyl]-4-methyl oxazolium bromide	0
VIII. 3-Benzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride	38
IX. 3-α-Methylbenzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride	0
X. 3,4-Dimethyl-5-(2-hydroxyethyl) thiazolium chloride	2
XI. 3-Cyanomethyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride	5
XII. 3-*o*, *m*, *p*-Nitrobenzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chlorides	Trace
XIII. 3-(3-Aminopropyl)-4-methyl thiazolium chloride	0
XIV. 3-(4-Aminobutyl)-4-methyl thiazolium chloride	0
XV. Dihydrothiamine	0
XVI. Tetrahydrothiamine	0
XVII. 4-Amino-5-aminomethyl-2-methyl pyrimidine	0

(XII) and the γ- and δ-aminoalkyl analogues XIII and XIV theoretically should work as catalysts. However, it is apparent from the color of the reaction mixtures that these compounds are not stable under the test conditions and little can be said about their catalytic activities.

As would be expected from Breslow's mechanism, reduction of the carbon-nitrogen double bond of the thiazolium ring causes complete loss of activity (dihydro- and tetrahydrothiamine). Two compounds in which the sulfur of the thiazolium ring is replaced by oxygen (oxazolium salts) were tested. One of these (*O*-heterothiamine) is otherwise identical with thiamine; the second (VII) lacks a group on C-5 of the thiazolium ring. Both compounds appear to be completely inactive as catalysts although the result with *O*-heterothiamine was obscured by its decomposition (see section on testing procedure). The inactivity of oxazolium salts probably results from their reaction with base to form inactive pseudo bases. Titrations indicate that this occurs at a low pH with a pK_a of about 5.8 for both of the oxazolium compounds ($K = [H^+][\text{pseudo base}]/[\text{oxazolium ion}]$).

The large difference in activity between the 3-methyl and 3-benzyl salts (X and VIII) is surprising. Breslow (13) suggests that the difference in inductive effects of the methyl and benzyl groups is responsible. As evidence Breslow compares the pK_a of methyl amine (10.6) with that of benzyl amine (9.3). We find the pK_a of the aminomethyl group of 4-amino-5-aminomethyl-2-methyl pyrimidine (XVII) to be 8.4.[2] Thus the inductive effect of the aminopyrimidine group in lowering the basicity of the aminomethyl group is even greater than that of a benzene ring, and thiamine is correspondingly more reactive than the benzyl analogue. However, the relationship is not really quantitative.

If the rate of reaction is governed by the extent of formation of the dipolar ion XVIII, the rate should be a maximum at the pK (average) of the thiazolium ring-opening reaction. This has been shown to be the case with thiamine and with the benzyl analogue (VIII) (4). The methyl thiazolium salt (X) could not be tested at its high pK value because of decomposition. The pK values for the ring-opening reactions of the three compounds, thiamine, the benzyl (VIII), and methyl (X) thiazolium salts at 25° are 9.25, 9.9, and 10.2, respectively. Thus, the effect of replacing a hydrogen on the *N*-methyl group of compound X with a phenyl or aminopyrimidyl group is to shift the optimal pH for reaction to a lower value.

At pH 8.7 to 9.0 the benzyl and methyl compounds exist almost completely in the thiazolium form but thiamine has been half converted to the inactive thiol form. A truer comparison of activities could be made at a pH of 8 or below where thiamine is over 99 per cent in the thiazolium form. We would expect thiamine to be about 5 times as active as the benzyl compound under these conditions. Thus one function of the aminopyrimidine group of thiamine may be to permit efficient catalysis in the physiological pH range.

Oxythiamine (IV), in which the amino group of thiamine has been replaced by a hydroxyl, is almost inactive at pH 8.4 to 8.9 as reported by Breslow (13). However, at pH 10.2 it has 20 per cent of the activity of thiamine. The pH for the ring-opening reaction is about 10.6. Titration of oxythiamine indicates that the oxypyrimidine group dissociates with a pK of about 8.2. Thus, under our test conditions the pyrimidine ring bears a negative charge and we would anticipate a marked decrease in reactivity at C-2 of the thiazolium group and the observed increase in the pK of the ring-opening reaction. Downes and Sykes (16) have attributed the low activity of oxythiamine to an actual hydrogen bond between the oxygen on the pyrimidine ring and C-2 of the thiazolium ring. However, it is unnecessary to postulate this unique hydrogen bond. There is also a possibility that the oxygen adds intramolecularly to the thiazolium ring as does the amino group of thiamine at a higher pH (7).

SUMMARY

1. A number of thiamine analogues have been synthesized and tested for catalytic activity in converting pyruvate to acetoin. The thiazolium ring is necessary for catalytic activity, but the pyrimidine ring is not.

2. Oxazolium salts, a 2,4-dimethyl thiazolium analogue of

[2] The pK_a value for the amino group attached directly to the ring in position 4 is 5.4. The change in the ultraviolet spectrum accompanying this first dissociation confirms that the 4-amino group is involved.

thiamine, pyrithiamine, dihydrothiamine, and tetrahydrothiamine are inactive.

3. The relative catalytic activities and pH optima of several thiazolium salts are discussed. The results are in harmony with the theory of Breslow in which a thiazolium dipolar ion is an intermediate.

REFERENCES

1. Mizuhara, S., Tamura, R., and Arata, H., *Proc. Japan Acad.*, **27**, 302 (1951).
2. Takeuchi, Y., Kyuichiro, O., Hayakawa, S., and Mizuhara, S., *J. Biochem. Tokyo*, **42**, 93 (1955).
3. Koffler, H., and Krampitz, L. O., *Bacteriol. Proc.*, 113 (1955).
4. Yatco-Manzo, E., Roddy, F., Yount, R. G., and Metzler, D. E., *J. Biol. Chem.*, **234**, 733 (1959).
5. Breslow, R., *Chem. & Ind.* London, B. I. F. Review, R 28 (1956).
6. Clarke, H. T., *J. Am. Chem. Soc.*, **66**, 652 (1944).
7. Maier, G. D., and Metzler, D. E., *J. Am. Chem. Soc.*, **79**, 4386 (1957).
8. Livermore, A. H., and Sealock, R. R., *J. Biol. Chem.*, **167**, 699 (1947).
9. Cornforth, J. W., and Cornforth, R. H., *J. Chem. Soc.*, **93** (1953).
10. Bonvicino, G. E., and Hennessy, D. J., *J. Am. Chem. Soc.*, **79**, 6325 (1957).
11. Sarver, H., Ph. D. Thesis, Iowa State College, 1948.
12. Watanabe, A., and Asahi, Y., *Yakugaku Zasshi*, **77**, 153 (1957).
13. Breslow, R., *J. Am. Chem. Soc.*, **80**, 3719 (1958).
14. Fry, K., Ingraham, L. L., and Westheimer, F. H., *J. Am. Chem. Soc.*, **79**, 5225 (1957).
15. Breslow, R., *Chem. & Ind.* London 893 (1957); *J. Am. Chem. Soc.*, **79**, 1762 (1957).
16. Downes, J. E., and Sykes, P., *Chem. & Ind.* London, 1095 (1957).
17. Ugai, T., Tanaka, S., and Dokawa, S., *Yakugaku Zasshi*, **63**, 296 (1943).
18. Yount, R. G. and Metzler, D. E., *Federation Proc.*, **17**, 341 (1958).

Studies on the Biosynthesis of Glucosamine in the Intact Rat

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Little is known about the biosynthesis of glucosamine in mammalian organisms. Previous studies have indicated the presence of enzymes in several mammalian tissues capable of synthesizing glucosamine (1, 2). Moreover, there is evidence that glucose, without scission of its carbon chain, may serve as the precursor of glucosamine in the intact animal (3, 4), as in bacteria (5, 6). However, except for studies on the mucopolysaccharides of skin (7, 8), no information is available on the rate of turnover of glucosamine in mammalian tissues. It would be of special interest to have information concerning the rate of turnover and the site of synthesis of the serum glucosamine, which is present in large concentrations and is apparently entirely protein-bound. As a major carbohydrate component of the serum glycoproteins, it undergoes wide fluctuations in concentration in a variety of physiological and pathological states (9).

In the present study a comparison was made of the incorporation of radioactivity from glucose-C^{14} into the protein-bound glucosamine of various tissues of the intact rat. Of the tissues studied, liver and serum showed the most rapid synthesis of glucosamine from glucose, and a detailed analysis of the turnover of glucosamine in both of these tissues was made. Evidence is also presented that the liver is the primary site of synthesis of the serum glucosamine.

EXPERIMENTAL

Animals—Male albino rats of the Wistar strain, weighing between 235 and 300 gm., were used. All animals were fed Purina chow *ad libitum* before as well as during the experiment, up to the time of death. Each animal received a single intraperitoneal injection of 10 to 15 μc. of uniformly labeled glucose-C^{14} (2.08 μc. per mg.) in 2 ml. of 0.85 per cent sodium chloride. At various time intervals before death 0.2 ml. of blood was taken from the tail vein of each rat without anesthesia for the purpose of determining the blood glucose specific activity. The animals were killed at varying time intervals ranging from 45 minutes to 48 hours after the injection of the glucose-C^{14}. They were anesthetized lightly with ether and exsanguinated from the inferior vena cava. Various organs were then excised and placed on ice.

Isolation of Glucosamine and Glycogen from Tissues—The method to be described for the separation of the protein-bound glucosamine from the liver was also used to obtain the glucosamine of the kidneys, lungs, testes, and spleen. In the case of the liver, the abundant amount of glycogen present was also isolated for the purpose of determining its specific activity. The liver was divided into portions weighing a maximum of 5 gm., and each portion was treated separately throughout the following procedure.

The tissue was minced with scissors and then homogenized for about 4 minutes in 7 per cent ice-cold trichloroacetic acid (5 ml. per gm. of tissue). The homogenate was then centrifuged and the precipitate re-extracted twice more with trichloroacetic acid in a similar manner. The liver extracts were pooled and saved for the isolation of glycogen. The trichloroacetic acid precipitate was then suspended in 0.3 N trichloroacetic acid (3 ml. per gm. of tissue), heated in a capped centrifuge tube in a boiling water bath for exactly 8 minutes, and then rapidly cooled in an ice bath. Under these conditions, no glucosamine was released from the protein precipitate, although about 8 μmoles of anthrone-positive material per gm. of liver, which probably represent residual glycogen as well as weakly bound hexoses, were released. After this, the residue was separated by centrifugation, washed once more with cold 0.3 N trichloroacetic acid, and then washed with 95 per cent ethanol (6 ml. per gm. of tissue) in order to remove the trichloroacetic acid. The residual ethanol was removed from the precipitate over a steam bath. The dry material was then transferred to a 25-ml. glass-stoppered Pyrex volumetric flask and hydrolyzed for 10 hours in 3 N HCl (3 to 4 ml. per gm. of tissue) in a boiling water bath in order to release the glucosamine. After the hydrolysis the flask was made up to volume with water and the hydrolysate was filtered. A measured amount of the filtrate was then concentrated to dryness three times in a vacuum at 45° in order to remove most of the HCl. Columns of Dowex 50-X4 cation exchange resin (200 to 400 mesh) in the hydrogen form were prepared. The resin was washed according to Boas (10), and 20 ml. of a 1:1 weight per volume suspension of the resin in water were placed into a glass column of 1.6-cm. internal diameter. The residue obtained upon concentration of the hydrolysate was taken up in about 15 ml. of water and quantitatively transferred to one of these columns in order to separate the remaining neutral sugars from the glucosamine. The column was then washed with about 1300 ml. of distilled water, after which the glucosamine was eluted with 2.0 N HCl. The first 7 ml. of the eluate contained no amino sugar and were discarded. All of the amino sugar was recovered in the next 10 ml. of eluate. The hydrolysate was completely decolorized by passage through the Dowex column. With the above procedure less than 8 per cent of the tissue amino acids was present in the collected eluate.

In order to separate the glucosamine from the remaining amino acids and to form a derivative suitable for determining the radioactivity, the glucosamine was converted to the glucose phenylosazone (11). In the case of liver, which had been divided into 5-gm. portions up to this point, the Dowex 50 eluates were

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combined and concentrated in a vacuum and made up to a volume of 10 ml. The glucosamine of the other tissues was present in the 10-ml. of eluate collected from a single column. An aliquot was taken for the determination of the glucosamine, and a maximum of 20 μmoles of carrier glucosamine was added to the remainder. In the case of the liver, since there were already about 20 μmoles of glucosamine present in the eluates, there was only very slight dilution of radioactivity by the carrier. The phenylosazone was formed in 12 ml. of an acetate buffered solution (1.2 M sodium acetate: 0.4 M acetic acid) containing 1 mmole of phenylhydrazine hydrochloride and about 40 μmoles of glucosamine in capped centrifuge tubes, by heating for 11 hours in a boiling water bath. The osazone was then allowed to crystallize in the cold. It was washed four times with water and recrystallized from 50 per cent ethanol. Further recrystallization resulted in no change in specific activity. With this procedure a yield of 50 to 55 per cent was obtained after washing and recrystallization. Shorter periods of heating such as have been used for the formation of the phenylosazone from glucose directly (12) resulted in very poor yields, *e.g.* 20 per cent in 2.5 hours. The osazones formed from the tissue eluates decomposed at about 204°, as did the osazones formed from pure glucose or glucosamine.

The liver glycogen was precipitated from the pooled cold trichloroacetic acid extracts by the addition of 1.2 volumes of 95 per cent ethanol. It was washed with 70 per cent ethanol and then hydrolyzed for 2.5 hours in 1 N H_2SO_4 in a boiling water bath. The hydrolysate was neutralized and an aliquot taken for the determination of the glucose released. To determine the specific activity, the remainder was used for the preparation of the glucose phenylosazone under the same conditions as for the glucosamine, but with heating for a period of only 2.5 hours.

Isolation of Glucosamine and Glucose from Blood—The blood collected from the animal at death was permitted to clot and about 3 to 4 ml. of serum were obtained. The serum proteins were precipitated by the addition of 15 volumes of 95 per cent ethanol and washed three times with 15 volumes of 95 per cent ethanol each time to remove the free serum glucose. After this, the ethanol was removed by evaporation on a steam bath, and the precipitate was hydrolyzed and passed through a Dowex 50 column in a manner identical to that described for liver and the other tissues. Similarly, the glucose phenylosazone was formed for the purpose of determining the radioactivity of the glucosamine.

The supernatant fluid from the ethanol precipitation of the serum proteins, which contained the free glucose, was evaporated to dryness in a vacuum desiccator. The solid material was dissolved in 10 ml. of water and a Somogyi filtrate (13) prepared from it. The 0.2-ml. samples of tail blood taken before the animals were killed were added to 4 ml. of water and the Somogyi filtrates were prepared. These filtrates were used for the analysis of glucose and the formation of the glucose phenylosazone after the addition of 10 mg. of carrier glucose.

Analyses—Glucose from glycogen and blood was determined by the Nelson-Somogyi method (14, 15). Total hexoses, except amino sugars, were determined with the anthrone reagent of Roe (16). The total hexosamines were determined on the Dowex 50 eluates by the Boas modification of the Elson-Morgan method (10). The percentage of glucosamine and galactosamine in a mixture of the amino sugars was determined according to the method of Roseman and Daffner (17), except for the following modifications. Because of the large concentration of nonhexosamine nitrogen in the Dowex eluates, the formation of the *N*-acetyl derivatives of the amino sugars was carried out by increasing the amount of acetic anhydride added to each tube to 0.15 ml. of a 12.5 per cent solution (volume for volume). The pH was maintained by the addition of 0.1 ml. of 4 N Na_2CO_3, instead of the saturated $NaHCO_3$ used in the original method. The heating period following acetylation was increased to 5 minutes. Because of the enhanced color formation obtained in the presence of borate buffer compared to carbonate buffer, chromogen formation was accomplished in the presence of 0.1 ml. of 0.8 M borate buffer, pH 9.1 (18), with a heating period of 7 minutes on a vigorously boiling water bath.

Paper Chromatography—Descending chromatograms were run in several solvent systems on Whatman No. 1 paper in order to identify the sugars present in the eluates from the Dowex 50 columns. In order better to identify the nature and proportion of the hexosamines present, they were converted to the corresponding pentoses as well as to their *N*-acetyl derivatives, which were subsequently chromatographed. The amino sugars were converted to pentoses by reaction with ninhydrin as described by Stoffyn and Jeanloz (19). Because of the presence of relatively large amounts of amino acids, the ninhydrin was added in large excess as a 4 per cent ninhydrin solution in 2 per cent pyridine to the sugar sample dissolved in a 0.1 M citrate buffer (pH 4.7). This reaction mixture was deionized by passage through a mixed bed ion exchange column (Amberlite MB-3), concentrated in a vacuum, and the pentoses chromatographed.

The *N*-acetyl derivatives of the amino sugars were prepared for chromatography in the same manner as for the colorimetric determination. Salts and amino acids were removed by passage through a mixed bed ion exchange column, followed by concentration in a vacuum, and chromatography of the *N*-acetyl derivatives on borate-treated paper as described by Cabib *et al.* (20).

All sugars were located on the paper chromatograms by the silver nitrate method of Trevelyan *et al.* (21). In addition, the aniline phthalate reagent (22) was used for the location of pentoses as well as hexoses, and amino sugars and their *N*-acetyl derivatives were specifically located with the Elson-Morgan method (23).

Measurement of Radioactivity—The radioactivity of the glucosamine, as well as of the glucose from either blood or glycogen, was determined on the glucose phenylosazone derivatives plated on stainless steel planchets in a windowless gas flow proportional counter (24). The activity of the injected glucose was also determined on a sample converted to the glucose phenylosazone. All counts were adjusted to 5.0×10^6 c.p.m. (about 10 μc.) injected into a 250-gm. rat.

Extensive studies were undertaken in order to exclude the possibility that the osazone formed from the glucosamine was contaminated with radioactivity from neutral hexoses, especially serum glucose and liver glycogen, or amino acids. As will be shown later, no neutral hexoses were present in the Dowex eluates by chromatographic analysis. However, in order to test this in a more sensitive manner, highly labeled glucose was added at varying steps of the isolation of the glucosamine from liver and serum. In the case of serum, no activity was present in the glucosamine derivative when radioactive glucose was added either to the serum before precipitation to simulate the free glucose or to the protein precipitate before hydrolysis to simulate protein-bound neutral hexoses. For liver, the amount of neutral

hexoses remaining in the tissue after the hot trichloroacetic acid extraction was determined to be 5 to 7 μmoles per gm., either by the anthrone reaction directly on the protein precipitate or from the Dowex 50 effluent and wash after a relatively mild hydrolysis (0.8 N HCl for 4 hours at 100°). Glucose in that quantity and with a specific activity much higher than that of the glycogen from any of the experiments was added to the protein precipitate before hydrolysis. Under these conditions, no activity was present in the osazone from glucosamine. It must be pointed out, however, that if radioactive glucose in an amount equal to that present in the unextracted liver (about 250 μmoles per gm.) was added to the precipitate before hydrolysis, some radioactive contamination resulted despite passage through a Dowex 50 column. The removal of the glycogen before hydrolysis is therefore essential. The results of the experiments to be reported confirm these contamination studies, as the blood glucose and blood glucosamine activity changed in an inverse manner, and there was no correlation between liver glycogen and liver glucosamine activity. A mixture of highly radioactive alanine and serine was added to the protein precipitate before hydrolysis and also directly to the Dowex eluate before osazone formation with no resulting activity appearing in the glucosamine derivative.

RESULTS

Identification of Amino Sugars—In order to identify the amino sugars and to determine whether any other sugars were present, the Dowex 50 eluates were chromatographed in a variety of systems. When the eluates from liver and serum were run in *n*-butanol-ethanol-water, 4:1:1, (40 hours) (19), only a single spot was found, corresponding to either glucosamine or galactosamine. After treatment with ninhydrin, the two amino sugars could be well resolved as their pentose derivatives: arabinose, $R_{glucose}$ 1.37; lyxose, $R_{glucose}$ 1.69. In the chromatograms of either serum or liver, only a single spot was present, corresponding to the arabinose standard or the glucosamine standard treated similarly with ninhydrin. Kidney and serum were chromatographed in a pyridine-ethyl acetate-water-glacial acetic acid system, 5:5:3:1, as described by Fischer and Nebel (25), in which the glucosamine standard migrated 28.2 cm. from the origin and the galactosamine standard, 25.3 cm. in 30 hours. For both tissues only a single spot corresponding to the glucosamine was observed. After acetylation, the eluates from liver and serum were chromatographed on borate-treated paper in ethyl acetate-pyridine-water, 2:1:2 (20) for 12 hours. In this system, the *N*-acetylglucosamine migrated 24.7 cm. and the *N*-acetylgalactosamine standard, 20.2 cm. during that time. Again, only a single spot corresponding to *N*-acetylglucosamine appeared in both tissues. This excludes the presence not only of protein-bound galactosamine in detectable amounts, but also of protein-bound mannosamine, of which the acetylated form has been reported by Comb and Roseman (26) to be clearly separable in this system. No other sugars were found in any of the chromatographic analyses.

When the protein-bound amino sugar composition of several rat tissues was analyzed by the method of Roseman and Daffner, it was shown, in agreement with the chromatographic results, that liver and serum contained no significant amounts of galactosamine (Table I). Moreover, only very small amounts of galactosamine were present in the other tissues. One is therefore well justified in referring to the amino sugar fraction of liver and serum as glucosamine. Moreover, the radioactivity measured in all of the tissues was due solely to glucosamine, since galactosamine in the small amounts present was found not to yield any galactosazone.

An analysis of the cold trichloroacetic acid extracts showed that all except 10 to 15 per cent of the glucosamine of liver is protein-bound. In serum, moreover, all of the glucosamine was protein-bound and was precipitated with the high concentration of ethanol used.

Incorporation of Radioactivity into Liver and Serum Glucosamine—In Table II the specific activity and total activity of liver and serum protein-bound glucosamine are shown in rats killed at varying time intervals after the injection of a tracer dose of glucose-C^{14}. The highest activity in liver glucosamine is already reached in animals killed in 1.5 hours. Serum glucosamine specific activity, on the other hand, reaches its peak by 3.75 hours. From the ratio of liver glucosamine specific activity to serum glucosamine specific activity (Table II), it may be seen that the serum is at first much less active than the liver, has about equal activity at 3.75 hours, and is more active than the liver at all times thereafter. This is graphically demonstrated in Fig. 1. It may be noted that there is little variation in the amount of glucosamine of the different livers. The serum protein-bound glucosamine pool was calculated from the glucosamine level and an assumed serum volume of 4 per cent of body weight.

Incorporation of Radioactivity into Liver Glycogen—In order to be able to compare the activity in liver glucosamine with a more familiar substance, the activity of liver glycogen at various times is shown in Table II. Contrary to the activity of the glucosamine, which is quite constant at any one time, the glycogen activity varies widely among animals even at the same time and shows no definite course with time. It may be noted that there is no correlation between the activity of the glycogen and that of the glucosamine. This is well demonstrated in Rat 15 (Table II) killed at 18 hours, which had a highly labeled glycogen, and yet showed the expected decline in the glucosamine activity.

Incorporation of Radioactivity into Glucosamine of Other Tissues—The activity of the protein-bound glucosamine of several other organs at various times was also determined (Table III). It may be seen that none of these reaches the early high activity present in liver glucosamine. The spleen has the highest specific activity of these organs, possibly because of its large blood content.

Incorporation of Radioactivity into Glucosamine after Injection of Uniformly Labeled Fructose-C^{14}—An attempt was made to study further the relationship of the liver and serum glucosamine by injecting with C^{14}-labeled fructose, which in the intact animal

TABLE I

Glucosamine content of total protein-bound hexosamine in rat tissues

The values are expressed as per cent of total hexosamine as determined by the differential colorimetric method. Difference between each value and 100 per cent represents the galactosamine fraction.

	Liver (14)*	Serum (15)	Kidney (5)	Lung (4)	Testes (2)
Mean	96	101	90	87	95
Standard error	±4.9	±4.1	±6.8	±9.1	±4.0

* The figures in parentheses indicate the number of animals.

TABLE II

Radioactivity in liver glucosamine, serum glucosamine, and liver glycogen after glucose-C^{14} injection

All counts adjusted to 5.0 × 10^6 c.p.m. injected into a 250-gm. rat.

Rat No.	Time	Liver glucosamine			Serum glucosamine*		Liver specific activity/ serum specific activity	Liver glycogen	
		Specific activity	Quantity†	Total activity	Specific activity	Total activity		Quantity†	Total activity
	hrs.	*c.p.m./ μmole*	*μmoles*	*c.p.m.*	*c.p.m./ μmole*	*c.p.m.*		*μmoles glucose equivalents*	*c.p.m.*
9	0.75	38.3	21.3	817	9.4	472	4.05	2,650	1,950
5	1.5	54.3	20.6	1,130	21.9	1,092	2.48	2,470	44,800
21	1.5	56.8	19.7	1,122	22.2	1,110	2.56	1,765	3,555
24	1.5	60.2	20.3	1,220	22.0	1,100	2.73	1,920	15,050
4	3.75	56.2	22.1	1,242	58.0	2,900	0.97	1,505	84,100
13	3.75	38.7	23.7	917	48.4	2,420	0.80	1,400	3,500
18	3.75	41.9	21.6	905	43.6	2,180	0.96	2,350	14,320
1	5.0	36.3	19.9	723	38.7	1,935	0.94	1,810	10,720
10	7.5	28.9	19.8	572	35.3	1,768	0.82	843	451
14	12.0	23.3	22.4	521	32.0	1,600	0.73	1,045	1,755
19	12.0	22.8	23.8	543	29.0	1,450	0.79	738	1,190
25	12.0	23.7	22.3	528	30.8	1,530	0.77	819	6,860
15	18.0	18.1	21.5	389	25.3	1,265	0.72	3,200	124,500
16	30.0	12.9	24.5	317	16.6	830	0.78	649	364
17	48.0	10.4	22.9	238	12.7	635	0.80	2,430	1,555
23‡	3.75	70.3	22.3	1,570	72.2	3,610	0.98	1,900	7,050

* Mean serum glucosamine level: 4.99 ± 0.20 μmoles per ml. Total activity calculated from a mean pool of 50 μmoles per 250-gm. rat, with the serum volume assumed to be 4 per cent of body weight.

† Quantity refers to the total μmoles in the liver of a 250-gm. rat.

‡ Fructose-C^{14} injected instead of glucose-C^{14} under the same conditions.

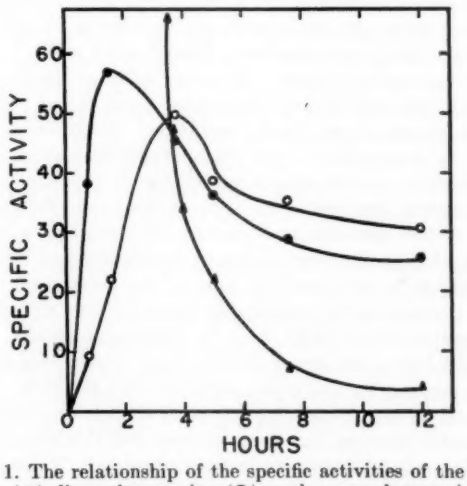

FIG. 1. The relationship of the specific activities of the serum glucose (▲), liver glucosamine (●), and serum glucosamine (○) after the injection of glucose-C^{14}. The mean specific activities for each time are plotted as c.p.m. per μmole of glucose or glucosamine. All values are adjusted to 5.0 × 10^6 c.p.m. injected into a 250-gm. rat.

TABLE III

Radioactivity in glucosamine of rat tissues after glucose-C^{14} injection

All counts adjusted to 5.0 × 10^6 c.p.m. injected into a 250-gm. rat. Total activity refers to the total c.p.m. in the glucosamine of the tissues of a 250-gm. rat. The mean ± the standard error of the glucosamine content of the tissues in μmoles per 250-gm. rat was kidney, 10.8 ± 0.4; testes, 4.48 ± 0.19; lung, 5.74 ± 0.47; spleen, 1.35 ± 0.09.

Rat No.	Time	Kidney		Lung		Testes		Spleen	
		Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
	hrs.	*c.p.m./ μmole*	*c.p.m.*	*c.p.m./ μmole*	*c.p.m.*	*c.p.m./ μmole*	*c.p.m.*	*c.p.m./ μmole*	*c.p.m.*
5	1.5	7.9	91	10.1	42	6.0	24.3	25.0	41.7
4	3.75	14.8	157	36.1	250	16.6	73.6	48.7	70.3
13	3.75	17.1	211						
1	5.0	12.3	133	16.9	101	13.7	70.1	29.3	38.3
10	7.5	15.0	143	20.3	110	14.4	60.3	31.1	34.9
14	12.0	16.0	177						
16	30.0	11.3	130						

appears to be utilized preferentially by the liver (27). For that purpose uniformly labeled fructose-C^{14} was injected into a rat under the same conditions used for glucose-C^{14}, and the animal was killed 3.75 hours thereafter. The ratio of specific activity of liver and serum glucosamine was found to be the same as after the injection of glucose. No significance can be attached to the absolute increase in these specific activities, since the specific activity of the fructose injected would undergo less dilution than that of the glucose injected.

DISCUSSION

The relationship between the specific activities of serum glucose, liver glucosamine, and serum glucosamine is shown in Fig. 1, in which the mean values of these functions are plotted for the various times up to 12 hours after the injection of the tracer dose of uniformly labeled glucose-C^{14}. From the graph it becomes apparent that the liver and serum glucosamine meet the criteria for the precursor-product relationship as formulated by Zilversmit *et al.* (28), namely, the specific activity of the liver glucosamine is initially greater than that of the serum glucosamine; when the specific activity of the serum glucosamine reaches its maximum, it is equal to that of the liver glucosamine; thereafter, the specific activity of the serum glucosamine is greater than that of the liver glucosamine. This relationship suggests that no other important sites for the synthesis of the serum protein-bound glucosamine exist, which would be consistent with the relatively low activity in the glucosamine of the other organs measured. Also, the observation that the ratio of liver and serum glucosamine specific activity is the same after the injection of either fructose-C^{14} or glucose-C^{14} is consistent with this idea that the liver is the site of synthesis of the serum glucosamine.

From Fig. 1 it may be noted that the serum glucose curve crosses the liver glucosamine curve to the right of its maximum. This would suggest that a simple product-precursor relation such as that described by Zilversmit *et al.* (28) is not adequate to explain the relationship between the serum glucose and the liver glucosamine. However, the curves for the serum glucose, liver glucosamine, and serum glucosamine appear to fit a model described by Russell (29), as well as by Barnum and Huseby (30).

In such a system the specific activity of the liver glucosamine would be determined not only by synthesis from the serum glucose, but also by a return of molecules from the serum glucosamine pool. This system could be described as follows.

$$\text{Serum glucose} \xrightarrow{v_1} \text{Liver glucosamine} \underset{v_3}{\overset{v_2}{\rightleftharpoons}} \text{Serum glucosamine} \xrightarrow{v_4?}$$

This model requires that the maximal specific activity of the liver glucosamine be reached before its curve crosses that of the serum glucose, which is considered its precursor, and that the maximal specific activity of the serum glucosamine occur only after both the liver and serum glucosamine have crossed the curve of the serum glucose (29). It may be seen from Fig. 1 that the data are consistent with this scheme, and moreover, such a system is physiologically quite credible.

By formulating a set of equations, an attempt has been made to calculate the rates of the above reactions from the curves shown in Fig. 1. In the equations to be used in this discussion, the following symbols will be employed: sA, sB, sC, the specific activities of the serum glucose, liver glucosamine, and serum glucosamine, respectively; B and C, the amounts of glucosamine present in liver and serum respectively; B* and C*, the amounts of radioactive glucosamine present in liver and serum; t_1 and t_2, $time_1$ and $time_2$, between which the calculations were made. The rate of conversion of liver glucosamine to serum glucosamine, v_2, may be calculated from the formula of Zilversmit *et al.* (28), which describes a simple product-precursor relationship.

$$v_2 = \frac{(sC_2 - sC_1)C}{\int_{t_1}^{t_2} sB\,dt - \int_{t_1}^{t_2} sC\,dt} \tag{1}$$

The denominator of the above fraction is obtained from the area between the liver and serum glucosamine curves between $time_1$ and $time_2$.

For the calculation of v_1, the rate of synthesis of liver glucosamine from serum glucose, and v_3, the rate of return of glucosamine from serum to liver, the following equations may be used.

$$\frac{dB^*}{dt} = v_1 sA + v_3 sC - (v_2 + v_4)sB \tag{2}$$

In a steady state $v_2 + v_4$ equals $v_1 + v_3$. By making this substitution and replacing B* by BsB, the following equation is obtained.

$$B\frac{dsB}{dt} = v_1(sA - sB) - v_3(sB - sC) \tag{3}$$

Integration of this equation gives

$$B(sB_2 - sB_1) = v_1\left[\int_{t_1}^{t_2} sA - \int_{t_1}^{t_2} sB\right]dt - v_3\left[\int_{t_1}^{t_2} sB - \int_{t_1}^{t_2} sC\right]dt \tag{4}$$

The integrals in this equation may be obtained from the areas between the serum glucose and liver glucosamine curves from $time_1$ to $time_2$ and between the liver glucosamine and serum glucosamine curves from $time_1$ to $time_2$, respectively.

At the maximum of the liver glucosamine curve, $\frac{dsB}{dt} = 0$, and, as may be seen from Equation 3, the following relationship must exist.

$$\frac{v_1}{v_3} = \frac{sB_m - sC_m}{sA_m - sB_m} \tag{5}$$

where m refers to the time at which the liver glucosamine reaches its maximal specific activity. From these two equations in two unknowns it should theoretically be possible to calculate both v_1 and v_3.

When v_2 is calculated from the curves between 45 and 90 minutes, with use of the mean serum glucosamine value of 50 μmoles, a rate of 25.6 μmoles per hour is obtained. When v_1 and v_3 were similarly calculated from the curves between 45 and 90 minutes, with the 90-minute value used as the maximum of the liver glucosamine specific activity, a rate of 0.68 μmole per hour was obtained for v_1 and 16.8 μmoles per hour for v_3. These values for v_1 and v_3 are approximations, since it is not possible to localize the maximum precisely. However, values of the same order of magnitude are obtained when other times in the vicinity of 90 minutes are chosen for the maximum. Since the values obtained for v_1 are always very much smaller than those of v_2 or v_3, indicating that the rate of interchange of glucosamine molecules between liver and serum is much more rapid than the synthesis of new glucosamine molecules from glucose, v_1 may be calculated in an alternate manner by considering liver and serum glucosamine to be essentially part of the same pool. Fig. 2 shows the relationship between the serum glucose curve and the calculated specific activity curve for this common pool. It may be noticed that the serum glucose curve crosses this common pool curve near its maximum, indicating that the steps between liver and serum glucosamine are rapid enough to consider the relationship between the serum glucose and this common glucosamine pool as a simple product-precursor one. V_1 may therefore be calculated from the curves in Fig. 2 with the same equation as has been employed for the liver and serum glucosamine relationship (Equation 1). Such a calculation carried out between 45 and 90 minutes gives a value for v_1 of 0.89 μmole per

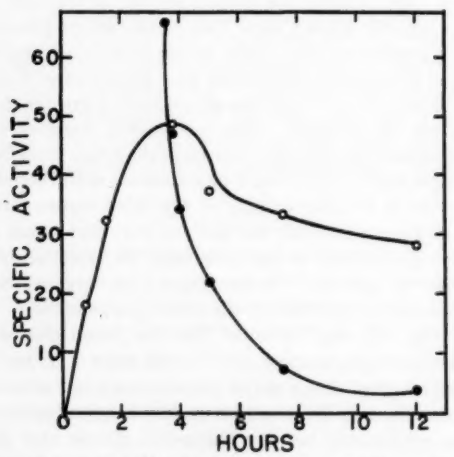

FIG. 2. The relationship of the specific activities of the serum glucose (●) and the glucosamine of the liver and serum considered as a common pool (○). Values are expressed as in Fig. 1.

hour. When this value for v_1 is substituted into Equation 4, a value of 26.3 μmoles per hour is obtained for v_3. This value for v_3 is probably more exact than that obtained from Equations 4 and 5, since it does not depend on the exact localization of the maximum of the liver glucosamine. When this value for v_3 is compared with v_2, it may be seen that they are almost identical, indicating that v_5 is either small or nonexistent. The turnover time for serum glucosamine, $\frac{C}{v_2}$, is 1.95 hours. The turnover time for liver glucosamine, $\frac{B}{v_1+v_3}$, with 0.89 and 26.3 used for v_1 and v_3, is 0.79 hours. The time required to replace the liver glucosamine by synthesis of new molecules from glucose, $\frac{B}{v_1}$, is 24.2 hours, whereas the time required to replace the serum glucosamine by synthesis from glucose, $\frac{C}{v_1}$, is 56.2 hours. These values are summarized in Table IV.

From these calculations it may be seen that the turnover time of both serum and liver glucosamine is extremely rapid and many fold faster than the time required for synthesis of new molecules from glucose. It remains open to speculation to what extent this rapid interchange of protein-bound glucosamine between liver and serum represents a breakdown and resynthesis of the glycoprotein molecules with a reutilization of most of the glucosamine, or merely a transfer back and forth between liver and serum of the same glycoprotein molecules.

Many values cited in the literature for turnover time of biological compounds are based on calculations made from radioactive decay of the compound plotted as the logarithm of the specific activity *versus* time. It is obvious from the above considerations that such a calculation would be quite erroneous in the present system because of the large extent of recycling of glucosamine molecules between liver and serum. However, in order to compare the turnover time of liver and serum glucosamine to turnover times of compounds calculated in this manner, the logarithm of the specific activity was assumed to be linear up to 12 hours and was plotted against time by means of the method of least squares. Turnover time was calculated from the half-time by multiplying it by 1.44 (31). The half-times obtained from these decay plots were 8.9 hours for liver glucosamine and 14.0 hours for serum glucosamine; the turnover times were calculated as 12.8 hours and 20.1 hours for the liver and serum glucosamine, respectively. These values may be compared with a half-time of 1.6 to 2.3 days determined from the decay of the glucosamine of hen egg ovomucoid (4) and with a half-time of 3.4 days determined for the glucosamine of the hyaluronic acid of the skin of rabbits (8).

The rapid turnover of glucosamine noted from the calculations of the present study is consistent with the work on the α_1 acid glycoprotein in guinea pigs by Boström *et al.* (32), who found that the peak of the glucosamine specific activity had occurred some time before 12 hours, when they made their first determination. They estimated from the decay of the specific activity of the glycoprotein that its half-time is about 1 to 2 days, which would be considerably longer than the half-time of the total serum protein-bound glucosamine determined in the present study.

TABLE IV

Metabolism of liver and serum glucosamine

All values expressed per 250-gm. rat.

Tissue	Tissue glucosamine pool	Turnover rate	Turnover time	Rate of synthesis from glucose	Synthesis time from glucose	Half-time from decay
	μmoles	*μmoles/hr.*	*hrs.*	*μmoles/hr.*	*hrs.*	*hrs.*
Liver.....	21.5	27.2	0.79	0.89	24.2	8.9
Serum.....	50.0	25.6	1.95	0.89	56.2	14.0

The site of formation of the serum glycoproteins has been a subject of some interest, and it has been proposed by some that depolymerization of ground substance of connective tissue may give rise to these glycoproteins (9). Since the glycoproteins are a heterogeneous group, it is not necessary that there be a single site of synthesis. As judged from the data of the present study, dealing with the relationship between the liver and serum protein-bound glucosamine, it appears that the bulk of the serum glycoproteins is synthesized in the liver. This is consistent with the study of Werner (33), who concluded that the liver was the site of glycoprotein formation on the basis of experiments showing that the increase in protein-bound glucosamine after bleeding in rabbits no longer occurred if the liver had been damaged previously with phosphorus or benzene. The subnormal levels of seromucoid seen in human subjects with parenchymatous liver disease are also in agreement with such a view (34).

The rapid transfer of glucosamine between liver and serum would suggest that alterations in that compound measured in the blood would reflect changes in the metabolism of glucosamine by the liver itself.

SUMMARY

The metabolism of protein-bound glucosamine has been studied in various tissues of the intact rat with the aid of tracer doses of uniformly labeled glucose-C^{14}. Specific activity-time curves for serum glucose, liver glucosamine, and serum glucosamine were drawn. From these it appeared that the liver is the primary site of synthesis of the serum glucosamine. Moreover, a very rapid interchange of the glucosamine between liver and serum was calculated to take place, resulting in a turnover time of 0.8 hour for the liver and 2 hours for the serum glucosamine. The rate of synthesis of glucosamine molecules in liver from glucose was calculated to be about 0.9 μmole per hour, in a 250-gm. rat.

The synthesis of the protein-bound glucosamine from glucose in the other organs studied, namely, kidney, lung, testes, and spleen, was shown to be well below that of the liver.

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REFERENCES

1. POGELL, B. M., AND GRYDER, R. M., *J. Biol. Chem.*, **228**, 701 (1957).
2. LELOIR, L. F., AND CARDINI, C. E., *Biochim. et Biophys. Acta*, **20**, 33 (1956).
3. BECKER, C. E., AND DAY, H. G., *J. Biol. Chem.*, **201**, 795 (1953).
4. RIEDER, S. V., AND BUCHANAN, J. M., *J. Biol. Chem.*, **232**, 951, 959 (1958).

5. Roseman, S., Moses, F. E., Ludowieg, J., and Dorfman, A., *J. Biol. Chem.*, **203**, 213 (1953).
6. Topper, Y. J., and Lipton, M. M., *J. Biol Chem.*, **203**, 135 (1953).
7. Schiller, S., Mathews, M. B., Goldfaber, L., Ludowieg, J., and Dorfman, A., *J. Biol. Chem.*, **212**, 531 (1955).
8. Schiller, S., Mathews, M. B., Cifonelli, J. A., and Dorfman, A., *J. Biol. Chem.*, **218**, 139 (1956).
9. Winzler, R. J., *Methods of Biochem. Anal.*, **2**, 279 (1955).
10. Boas, N. F., *J. Biol. Chem.*, **204**, 553 (1953).
11. Tiemann, F., *Ber. deut. chem. Ges.*, **19**, 49 (1886).
12. Garard, I. D., and Sherman, H. C., *J. Am. Chem. Soc.*, **40**, 955 (1918).
13. Somogyi, M., *J. Biol. Chem.*, **160**, 69 (1945).
14. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
15. Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945).
16. Roe, J., *J. Biol. Chem.*, **212**, 335 (1955).
17. Roseman, S., and Daffner, I., *Anal. Chem.*, **28**, 1743 (1956).
18. Reissig, J. L., Strominger, J. L., and Leloir, L. F., *J. Biol. Chem.*, **217**, 959 (1955).
19. Stoffyn, P. J., and Jeanloz, R. W., *Arch. Biochem. Biophys.*, **52**, 373 (1954).
20. Cabib, E., Leloir, L. F., and Cardini, C. E., *J. Biol. Chem.*, **203**, 1055 (1953).
21. Trevelyan, W. E., Procter, D. P., and Harrison, J. S., *Nature*, **166**, 444 (1950).
22. Partridge, S. M., *Nature*, **164**, 443 (1949).
23. Partridge, S. M., *Biochem. J.*, **42**, 238 (1948).
24. Karnovsky, M. L., Foster, J. M., Gidez, L. I., Hagerman, D. D., Robinson, C. V., Solomon, A. K., and Villee, C. A., *Anal. Chem.*, **27**, 852 (1955).
25. Fischer, F. G., and Nebel, H. J., *Z. physiol. Chem.*, **302**, 10 (1955).
26. Comb, D. G., and Roseman, S., *J. Am. Chem. Soc.*, **80**, 497 (1958).
27. Hers, H. G., *J. Biol. Chem.*, **214**, 373 (1955).
28. Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, **26**, 325 (1943).
29. Russell, J. A., *Perspectives in Biol. Med.*, **1**, 138 (1958).
30. Barnum, C. P., and Huseby, R. A., *Arch. Biochem.*, **29**, 7 (1950).
31. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **176**, 209 (1948).
32. Boström, H., Rodén, L., and Yamashina, I., *J. Biol. Chem.*, **230**, 381 (1958).
33. Werner, I., *Acta Physiol. Scand.*, **19**, 27 (1949).
34. Greenspan, E. M., *Advances in Internal Med.*, **7**, 101 (1955).

The Role of Phosphorylation in Glucose Absorption from the Intestine of the Golden Hamster*

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The exact biochemical mechanism by which glucose is absorbed by the intestine against a concentration gradient is yet to be established. In 1933, Wilbrandt and Laszt (1) proposed the phosphorylation-dephosphorylation hypothesis for sugar absorption by the small intestine. In the same year Lundsgaard (2) suggested the same hypothesis for glucose reabsorption by the kidney tubules. Much of the evidence on which this hypothesis was originally based has since proved inadequate. On the other hand, conclusive evidence against the hypothesis is lacking.

The present study was designed to test the hypothesis of the phosphorylation of glucose during the transport process. Ashmore *et al.* (3) employed a method for calculating the quantity of glucose which was phosphorylated to glucose 6-phosphate by liver slices and subsequently hydrolyzed to glucose and inorganic phosphate. In a similar manner an attempt has been made in the present study to calculate the quantity of glucose arising from glucose 6-phosphate during the course of intestinal absorption of glucose by sacs of hamster intestine. The results obtained by such experiments indicate that only a small fraction of glucose transported passes through glucose 6-phosphate as an intermediate.

EXPERIMENTAL

Materials and Methods

Preparation of Tissue—Golden hamsters (80 to 150 gm.) fed Purina laboratory chow were used. The animal was killed by a blow on the head, the abdomen opened, and the entire small intestine washed out *in situ* with 0.9 per cent NaCl. The intestine was stripped from its mesentery and turned inside out with the use of a long probe. After washing the everted intestine carefully in 0.9 per cent NaCl, it was placed in a Petri dish that contained a modified Krebs-Henseleit (4) bicarbonate-sodium chloride medium with the following composition in mmoles: Na, 143; K, 6; Mg, 1.2; Ca, 1.3; Cl, 125; and HCO_3, 25. Tied sacs of intestine (about 200 mg. wet weight of tissue) were prepared in the manner previously described (5). From the gut of a single animal 2 to 8 sacs were prepared.

A measured volume (approximately 1 ml.) of bicarbonate-saline medium was injected into each sac (serosal solution). For the mucosal solution 3 or 5.0 ml. of bicarbonate-sodium chloride medium were used. The filled sacs were placed in 50-ml. flasks containing the mucosal solution. Flasks were gassed with 5 per cent CO_2 and 95 per cent O_2 and incubated with shaking for 1 hour at 37°. The final volume on the serosal side was determined by weighing the sac before and after emptying the contents. The final mucosal volume was calculated from the initial value and the change on the serosal side, assuming no tissue swelling or evaporation.

Radioactive Substrates—The radioactive substrates[1] employed were glucose-1-C^{14}, glucose-6-C^{14}, and galactose-1-C^{14}. Their initial specific activities were measured as the glucosazone or galactosazone. Counting techniques were as previously described (6).

Experimental Design—Three different studies were performed.

1. To estimate the contribution of the phosphogluconate oxidative pathway to gut metabolism, glucose-1-C^{14} or -6-C^{14} was present in both the mucosal and serosal solutions of a sac at a concentration of 200 mg. per 100 ml. Sacs from adjacent segments of intestine were used, one labeled with glucose-1-C^{14} and the other with -6-C^{14}. At the completion of the incubation, the sac was cut open and the mucosal and serosal solutions combined. An aliquot of this combined solution was discharged into sulfuric acid, the CO_2 evolved was trapped in sodium hydroxide, and precipitated as barium carbonate for assay. Total CO_2 in the flask, *i.e.* in the gas phase and the medium, was shown to be about 225 μmoles.

2. To estimate the extent of rearrangement of the glucose carbon skeleton during transport, glucose-6-C^{14} was placed at a concentration of 200 mg. per 100 ml. on the mucosal side and the glucose transported during the incubation was isolated as the glucosazone from the serosal solution with the aid of carrier glucose. The glucosazone was assayed and then degraded (7) to the 1,2-bisphenylhydrazone mesoxalaldehyde, which was also assayed. This latter compound contained carbons-1, -2, and -3 of the glucose molecule.

3. To estimate the quantity of glucose transported which had glucose 6-phosphate as an intermediate, sacs from adjacent segments were incubated in 5 ml. of bicarbonate-sodium chloride medium containing either galactose-1-C^{14} (100 mg. per 100 ml.) and nonradioactive glucose (200 mg. per 100 ml.), or nonradioactive galactose (100 mg. per 100 ml.) and glucose-1-C^{14} (200 mg. per 100 ml.). Bicarbonate-saline medium, 1 ml., (containing no sugar) was placed on the serosal side. At the completion of the incubation, the concentration of glucose in the mucosal and sero-

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[1] Glucose-1-C^{14} was purchased from Chicago-Nuclear Corporation, Chicago, Illinois; glucose-6-C^{14} from Volk Radiochemical Company, Chicago, Illinois; and galactose-1-C^{14} from the National Bureau of Standards, Washington, D. C.

sal solutions was estimated by determining reducing substance (8) before and after incubation with glucose oxidase. Galactose was taken as the reducing sugar unaffected by the enzyme, with a small correction for slow oxidation of the galactose. For the determination of radioactivity in CO_2 as described above 1 ml. of mucosal solution was taken.

In order to determine the activity of the glucose and galactose transported, 0.25 ml. of the serosal solution was placed on a 10-cm. line at the origin of a paper chromatogram. The chromatogram was developed for 40 hours in the descending direction with butanol-pyridine-water (6:4:3) (9). This solvent separates glucose from galactose, glucose running in the forward position. After drying, a thin strip of paper was cut from each side of the chromagram and sprayed with benzidine (10) to develop the sugar spots. By this method it was possible to determine the areas occupied by glucose and galactose in the untreated central portion of the paper. The forward one-half of the glucose band was eluted, the quantity of sugar determined (8), and the glucosazone was prepared with the aid of carrier glucose. A similar procedure was followed with the entire galactose band with carrier galactose added to facilitate the isolation of the galactosazone. An aliquot of the initial incubation medium was treated in the same way, and it was found that the specific activities of the substrates run on paper chromatograms and eluted varied no more than 10 per cent from the specific activity determined by preparation directly of the osazones. There was no contamination of nonradioactive glucose by galactose-1-C^{14} when it was isolated from solution by this chromatographic procedure. Furthermore, the osazones were counted, recrystallized, and counted a second time. Constant specific activity was found in all cases. It was previously found that by such recrystallization galactosazone could be separated from glucosazone (11).

Calculations—The μmoles of C^{14}-labeled glucose or galactose oxidized to CO_2, the μmoles of glucose-6-C^{14} incorporated into the mesoxalaldehyde, and the μmoles of galactose converted to glucose were calculated from (*a*) the specific activity of the substrates, (*b*) the specific activity of the metabolic products, and (*c*) the quantity of metabolic products present. Results were expressed as μmoles per 100 mg. of intestine wet weight per 60 minutes of incubation.

In the derivation of the quantity of glucose on the mucosal side converted to glucose on the serosal side via glucose 6-phosphate, it was assumed that glucose and galactose metabolism in the intestinal epithelial cell was similar to that in other mammalian cells and that most of the metabolism studied in the preparation occurred in the epithelial cells. That is, as shown in Fig. 1, it was assumed that in the metabolism of the intestine glucose and galactose had as their common intermediate glucose 6-phosphate, that carbon dioxide was formed from these substrates only after conversion to glucose 6-phosphate, and that glucose, once phosphorylated to glucose 6-phosphate, could be released as free glucose only directly from glucose 6-phosphate.

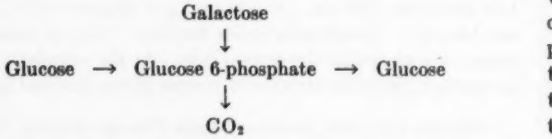

FIG. 1. Assumed interrelationships in the gut epithelium of glucose and galactose metabolism.

It was further assumed that glucose and galactose both contributed to the same glucose 6-phosphate pool and that the CO_2 collected served as a sampling of the contributions of each to the pool. Therefore, since both CO_2 and glucose arose from the same glucose 6-phosphate pool, the relative proportion of glucose and galactose converted to CO_2 was the same as the proportion of the two substrates converted to glucose, *i.e.*:

$$\frac{\text{Glucose to } CO_2}{\text{Galactose to } CO_2} = \frac{\text{glucose to glucose-6-PO}_4 \text{ to glucose}}{\text{galactose to glucose-6-PO}_4 \text{ to glucose}}$$

The quantity of glucose converted to glucose via glucose-6-PO_4 was then compared with the quantity of glucose that was transported during the experiment. This latter quantity was taken as that quantity of glucose found on the serosal side of the intestinal wall after incubation.

RESULTS

Since a knowledge of the extent of the conversion of radioactive glucose to CO_2 was required for the determination of glucose phosphorylation, preliminary studies were carried out on the effect of location along the gut on this conversion. Both glucose-1-C^{14} and glucose-6-C^{14} oxidation to CO_2 were studied at different locations of the gut to obtain the additional information as to the possible presence of the phosphogluconate oxidative pathway. Table I shows the results obtained in three animals, six sacs of tissue being prepared from each. In all cases but one, the CO_2 produced from glucose-1-C^{14} was more radioactive than that from glucose-6-C^{14}, suggesting the presence of the oxidative pathway.

One possible mechanism of glucose transport involving glucose 6-phosphate as an intermediate was investigated with glucose-6-C^{14}. If the mechanism for transport were the conversion of hexose to triose followed by the resynthesis of hexose, glucose-6-C^{14} entering the cell from the mucosal side would emerge on the opposite side as glucose-1-6-C^{14}. An experiment designed to test this possibility is shown in Table II. In each of the four sacs over 95 per cent of the initial glucose-6-C^{14} was absorbed from the mucosal side, an average of 3.5 mg. of the initial 6 mg. being recovered on the serosal side. The average final concentration gradient was 74-fold. The glucose transported to the serosal side was isolated at the end of incubation as the phenylosazone. This was oxidized to the 1,2-bisphenylhydrazone of mesoxalaldehyde which contained carbons-1, -2, and -3 of the original glucose molecule. Table II (Column 8) shows that no significant activity was found in the first three carbons (isolated as the mesoxalaldehyde). One further point of interest is that glucose emerging on the serosal side had about the same specific activity as that on the mucosal side initially (Columns 3 and 4). This indicates negligible dilution of the glucose in its passage through the tissue.

An attempt was next made to evaluate the role of the conversion of glucose to glucose 6-phosphate in the process of glucose absorption. The experiments were carried out in the presence of both glucose and galactose on the mucosal side of the intestinal wall; in one flask glucose was radioactive, while in the other galactose was radioactive. As there was some variation from one location of the gut to another, three consecutive segments were used; the first and third were incubated in one solution, and the second in the other. The average values for

TABLE I

Conversion of glucose-1-C^{14} and glucose-6-C^{14} to CO_2

From each of three hamsters 6 sacs were prepared and alternate sacs were incubated with either glucose-1-C^{14} or glucose-6-C^{14} at a glucose concentration of 200 mg. per 100 ml. The carbon dioxide formed from the labeled glucose is presented as μmoles of CO_2 per 100 mg. of intestine per 60 minutes of incubation.

	Upper jejunum			Mid gut			Lower ileum		
	Glucose-1-C^{14} (*a*)	Glucose-6-C^{14} (*b*)	Ratio (*a*)/(*b*)	Glucose-1-C^{14} (*c*)	Glucose-6-C^{14} (*d*)	Ratio (*c*)/(*d*)	Glucose-1-C^{14} (*e*)	Glucose-6-C^{14} (*f*)	Ratio (*e*)/(*f*)
Hamster 1	1.55	1.07	1.45	1.09	0.56	1.95	0.80	0.56	1.43
Hamster 2	0.84	0.50	1.68	0.59	0.39	1.51	0.76	0.48	1.58
Hamster 3	0.84	0.65	1.29	0.81	0.79	1.09	0.47	0.47	1.00
Mean	1.08	0.74	1.47	0.83	0.55	1.52	0.68	0.50	1.36

TABLE II

Possible conversion of glucose-6-C^{14} to glucose-1-6-C^{14} during intestinal transport

Glucose-6-C^{14} (200 mg. per 100 ml.) was placed in the mucosal incubation medium of 4 sacs from two hamsters. The specific activity of the glucose employed and of the glucose isolated from the serosal medium at the completion of the incubation (60 minutes) was determined as the glucosazone. The initial and serosal glucosazones were then degraded to their corresponding mesoxalaldehydes (which contain carbons 1, 2, and 3 of the glucose) and their activity was determined. Specific activity is given in c.p.m. per μmole of glucosazone or mesoxalaldehyde.

Hamster No.	Segment	Initial glucosazone specific activity (*a*)	Serosal glucosazone specific activity (*b*)	Initial specific activity (*b*) (100)/(*a*)	Initial mesoxalaldehyde specific activity (*c*)	Serosal mesoxalaldehyde specific activity (*d*)	Activity in mesoxalaldehyde (*d* − *c*) (100)/(*b*)
				%			%
1	Jejunum	3020	3360	111	65	53	0
1	Ileum	3020	3040	100	65	65	0
2	Jejunum	3520	3880	110	39	72	1
2	Ileum	3520	3900	111	39	0	0

the first and third segments were compared with that of the second. When radioactive galactose was present during glucose absorption, the glucose appearing on the serosal side contained some radioactivity. Glucose 6-phosphate was assumed to be intermediate in this interconversion. An estimate of the proportion of glucose passing through glucose 6-phosphate during transport is given in a representative experiment in Table III. First, the relative contribution of glucose and galactose to the glucose 6-phosphate pool was estimated by the ratio of μmoles of each sugar converted to CO_2. If glucose passed through this pool of glucose 6-phosphate during transport, the glucose appearing on the serosal side should be approximately the same specific activity as the pool. A sample calculation is given in Table III.

The results from six complete experiments are shown in Table IV. It is estimated that an average of 8.7 per cent of the glucose transported was phosphorylated at some point between mucosal and serosal sides. Whether this quantity of glucose was phosphorylated during actual active transport or during the metabolic maintenance processes of the cell cannot be stated.

To test whether the relative contributions of the two sugars to the glucose 6-phosphate pool changed with time, an experiment was carried out in which CO_2 was sampled at both 30 and 60 minutes. The ratios of CO_2 production from glucose and galactose at 30 minutes was similar to that at 60 minutes.

In all of these experiments a considerable portion of the glucose initially present on the mucosal side moved across the wall to the serosal side. Although there was initially no sugar on the serosal side, much of the sugar must have moved against a concentration gradient as the final concentration on the serosal side was an average of 7.3 times that on the mucosal side (average of 16 intestinal sacs) at the end of incubation. Galactose, on the other hand, moved across the gut wall only slowly and in 15 out of 16 cases its concentration on the serosal side was lower

TABLE III

Sample calculation of fraction of transported glucose which was phosphorylated*

Sacs were prepared from three adjacent segments of small intestine of a single hamster. The first and third segments were incubated with galactose-1-C^{14} (100 mg. per 100 ml.) in the presence of glucose (200 mg. per 100 ml.) and the second with galactose (100 mg. per 100 ml.) in the presence of glucose-1-C^{14} (200 mg. per 100 ml.). Values are given as μmoles per 100 mg. of tissue per 60 minutes of incubation.

Sac No.	Location	Unlabeled sugar	Labeled sugar	Sugar-C^{14} to CO_2	Galactose-1-C^{14} to glucose	Glucose transported
1	upper jejunum	glucose	galactose	0.12	0.15	9.68
2	mid jejunum	galactose	glucose	0.72		
3	low jejunum	glucose	galactose	0.06	0.06	7.60

* $$\frac{\text{Glucose-1-C}^{14}\text{ to CO}_2}{\text{Galactose-1-C}^{14}\text{ to CO}_2} = \frac{\text{Glucose (mucosal) to glucose (serosal) via Glucose-6-P}}{\text{Galactose (mucosal) to glucose (serosal) via Glucose-6-P}}$$

Substituting we have:

$$\text{Glucose (mucosal) to glucose (serosal) via glucose-6-P} = \frac{(0.72)\ 0.5\ (0.15 + 0.06)}{0.5\ (0.12 + 0.06)} = 0.84$$

Net glucose transported (average of Sacs 1 + 3) = 8.6

Per cent of glucose transported via glucose-6-P = (0.84/8.6)(100) = 10 per cent

TABLE IV

Fraction of transported glucose which was phosphorylated

Sacs of intestine were incubated with glucose and galactose on the mucosal side, one sugar or the other being radioactive. No sugar was on the serosal side. Incubation was for 1 hour at 37°. See Table III for sample experiment.

No.	Glucose to CO_2 / Galactose to CO_2	Galactose to glucose	Glucose to glucose via Glucose-6-P	Glucose transported	Transport sugar phosphorylated
					%
1	8.0	0.105	0.84	8.6	10
2	12.0	0.050	0.60	4.2	14
3	5.1	0.069	0.35	8.0	4
4	9.3	0.040	0.37	7.8	5
5	22.0	0.037	0.82	5.4	15
6	9.4	0.039	0.37	9.0	4
Average					8.7

TABLE V

Inhibition of galactose transport and metabolism by glucose

Sacs of intestine were incubated with 5 ml. of bicarbonate-sodium chloride containing 100 mg. per 100 ml. of galactose-1-C^{14} with or without glucose (200 mg. per 100 ml.) on the mucosal side. The serosal side contained no sugar. Galactose disappearing from the medium during incubation is indicated by "galactose utilized." All values are given as μmoles per 100 mg. of tissue per 60 minutes of incubation.

Animal No.	Location	Sugars present	Galactose transported	Galactose concentration gradient*	Galactose utilized	Galactose to CO_2
1	Upper ileum	Galactose	5.4	17.1	1.9	0.084
1	Lower jejunum	Galactose + glucose	1.1	0.65	0.29	0.032
2	Lower jejunum	Galactose	6.4	∞†	2.0	0.17
2	Upper ileum	Galactose + glucose	1.8	0.80	0.3	0.057

* Final concentration on serosal side/final concentration on mucosal.

† Final serosal concentration = 259 mg. per 100 ml.; final mucosal concentration = 0.

than that on the mucosal side at the termination of the incubation.

Glucose has a profound effect on both the transport rate and the utilization rate of galactose. This is illustrated in Table V. A very striking inhibition of galactose transport was noted with glucose (200 mg. per 100 ml.) at a concentration twice that of galactose (100 mg. per 100 ml.). In the presence of glucose, galactose was still moving down a concentration gradient at the time when, in the absence of glucose, a very large concentration gradient was developing. Also oxidation of galactose to CO_2 and galactose utilization was markedly decreased in the presence of glucose. Although the inhibition of galactose transport by glucose is well known (12, 13), the inhibition of utilization and CO_2 production has not previously been noted.

DISCUSSION

The phosphorylation-dephosphorylation hypothesis of sugar transport (1, 2) was based primarily on the observations that iodoacetate and phlorizin (thought at that time to be specific inhibitors of phosphorylation reactions) inhibited glucose absorption by the small intestine (1, 14–17) and kidney (18–20). Subsequent studies have shown that iodoacetate is not a specific inhibitor but reacts with the sulfhydryl groups of many enzymes. Furthermore, the primary site of action of phlorizin is apparently not on hexokinase or phosphatase but on oxidative pathways (21, 22). Additional evidence for the hypothesis was the finding that the kinase activity in the intestine for different sugars was proportional to their rate of intestinal absorption (23–25), although this was subsequently disputed (26). The accumulation of sugar phosphates within the epithelium which occurs during absorption (27–32) has also been taken as evidence for the hypothesis. However, many cells increase the concentration of intermediates of metabolism on the addition of utilizable sugars and the accumulation of sugar phosphates cannot be taken as conclusive evidence for their participation in the transport process itself (although it is consistent with the hypothesis). Alterations in the concentration of phosphatases in the kidney (33) and gut (34) in various states have also been used to support the hypothesis.

One variation of the general phosphorylation hypothesis involves the conversion of glucose to two triose phosphates followed by recondensation to hexose. This possibility was considered (35) as lactic acid was found during glucose and fructose absorption both *in vitro* (5, 36–38) and *in vivo* (39–41). Considerable evidence against this triose hypothesis, however, has been presented both for the intestine (35, 42, 43) and the kidney (44). The present experiments (Table II) provide further evidence that this mechanism does not occur in the small intestine of the hamster.

Two recent studies have offered what may be considered direct evidence against the general phosphorylation hypothesis. First, Sols (26) was unable to detect kinase activity for the sugars galactose and 3-*O*-methylglucose, two sugars transported by the small intestine against concentration gradients (35). However, indirect evidence suggests that galactose is phosphorylated. The hamster intestine utilizes considerable quantities of galactose (35), converts some to CO_2 (see Table V) and a small amount to lactic acid (37). Qualitative tests performed by Kjerulf-Jensen (30) on the phosphate esters accumulated in the gut epithelium of the rat during galactose absorption suggested the presence of a galactose phosphate. Although 3-*O*-methylglucose is not utilized by the intact rat (45) nor converted to lactic acid by the intestine (37), its phosphorylation by preparations of rat intestine has been reported (46). During 3-*O*-methylglucose absorption by the hamster intestine small amounts of a sugar phosphate can be isolated by ion exchange columns, which on elution from the column and treatment with phosphatase yield a sugar chromatographically identical with 3-*O*-methylglucose.[2]

The second observation inconsistent with this hypothesis is the finding of Crane and Krane (47) that both 1-deoxyglucose and 6-deoxyglucose are transported by sacs of hamster intestine. The assumption was made that these two sugars and glucose are transported by a common mechanism and that phosphorylation

[2] T. H. Wilson, unpublished experiments.

of hydroxyl groups on carbons other than 1 and 6 does not occur in the absorption mechanism. The assumptions appear reasonable, but await experimental verification.

The experiments presented in this paper add further weight of evidence against the participation of glucose 6-phosphate as an intermediate in the transport of glucose across the intestine, at least in the case of the hamster. It should be emphasized that a number of assumptions have been made in the calculations used and the conclusions must be considered tentative until a more direct method for testing the hypothesis is available.

SUMMARY

1. The conversion of glucose-1-C^{14} and glucose-6-C^{14} to CO_2 has been studied with sacs of hamster intestine *in vitro*. The presence of the phosphogluconate oxidative pathway was indicated by the greater CO_2 production from C-1 than C-6 labeled glucose.

2. The possibility of hexose conversion to triose followed by resynthesis of hexose by the intestine was tested. It was presumed not to occur as glucose-6-C^{14} absorbed from the mucosal side of the *in vitro* preparation did not show randomization on passage across the intestinal wall.

3. By incubating sacs of intestine in paired flasks in the presence of glucose and galactose, the quantity of glucose transported via glucose 6-phosphate was calculated. Only a small fraction of the absorbed glucose passed through the glucose 6-phosphate pool which had been labeled with radioactive galactose.

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REFERENCES

1. Wilbrandt, W., and Laszt, L., *Biochem. Z.*, **259**, 398 (1933).
2. Lundsgaard, E., *Biochem. Z.*, **264**, 209 (1933).
3. Ashmore, J., Cahill, G. F., Jr., Hastings, A. B., and Zottu, S., *J. Biol. Chem.*, **224**, 225 (1957).
4. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
5. Wilson, T. H., and Wiseman, G., *J. Physiol.*, **123**, 116 (1954).
6. Karnovsky, M. L., Foster, J. M., Gidez, L. I., Hagerman, D. D., Robinson, C. V., Solomon, A. K., and Villee, C. A., *Anal. Chem.*, **27**, 852 (1955).
7. Topper, Y. J., and Hastings, A. B., *J. Biol. Chem.*, **179**, 1255 (1949).
8. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
9. Jeanes, A., Wise, C. S., and Dimler, R. J., *Anal. Chem.*, **23**, 415 (1951).
10. Bacon, J. S. D., and Edelman, J., *Biochem. J.*, **48**, 114 (1951).
11. Landau, B. R., Hastings, A. B., and Zottu, S., *J. Biol. Chem.*, **233**, 1257 (1958).
12. Cori, C. F., *Poc. Soc. Ezptl. Biol. Med.*, **23**, 290 (1926).
13. Fisher, R. B., and Parsons, D. S., *J. Physiol.*, **119**, 224 (1953).
14. Nakazawa, F., *Tohoku J. Exptl. Med.*, **3**, 288 (1922).
15. Lundsgaard, E., *Biochem. Z.*, **264**, 221 (1933).
16. Wertheimer, E., *Arch. ges. Physiol. Pflüger's*, **233**, 514 (1934).
17. Donhoffer, S., *Arch. exptl. Pathol. Pharmakol.*, **177**, 689 (1935).
18. Poulsson, L. T., *J. Physiol.*, **69**, 411 (1930).
19. Shannon, J. A., Jolliffe, N., and Smith, H. W., *Am. J. Physiol.*, **102**, 534 (1932).
20. Walker, A. M., and Hudson, C. L., *Am. J. Physiol.*, **118**, 130 (1937).
21. Shapiro, B., *Biochem. J.*, **41**, 151 (1947).
22. Lotspeich, W. D., and Keller, D. M., *J. Biol. Chem.*, **222**, 843 (1956).
23. Hele, M. P., *Biochem. J.*, **55**, 857 (1953).
24. Hele, M. P., *Biochem. J.*, **55**, 864 (1953).
25. Bissegger, A., and Laszt, L., *Helv. Physiol. Acta*, **9**, C60 (1951).
26. Sols, A., *Biochim. et Biophys. Acta*, **19**, 144 (1956).
27. Laszt, L., and Sullmann, H., *Biochem. Z.*, **278**, 401 (1935).
28. Lundsgaard, E., *Z. physiol. Chem.*, **261**, 193 (1939).
29. Beck, L. V., *J. Biol. Chem.*, **143**, 403 (1942).
30. Kjerulf-Jensen, K., *Acta Physiol. Scand.*, **4**, 225 (1942).
31. Naito, Y., *J. Biochem.*, **36**, 131 (1944).
32. Nagasawa, S., *J. Biochem.* **44**, 399 (1957).
33. Marsh, J. B., and Drabkin, D. L., *J. Biol. Chem.*, **168**, 61 (1947).
34. Verzar, F., and Sailer, E., *Helv. Physiol. Acta*, **10**, 247 (1952).
35. Wilson, T. H., and Vincent, T. N., *J. Biol. Chem.*, **216**, 851 (1955).
36. Wilson, T. H., *Biochem. J.*, **56**, 521 (1954).
37. Wilson, T. H., *J. Biol. Chem.*, **222**, 751 (1956).
38. Newey, H., Smyth, D. H., and Whaler, B. C., *J. Physiol.*, **129**, 1 (1955).
39. Kiyasu, J. Y., Katz, J., and Chaikoff, I. L., *Biochim. et Biophys. Acta*, **21**, 286 (1956).
40. Kiyasu, J. Y., and Chaikoff, I. L., *J. Biol. Chem.*, **224**, 935 (1957).
41. Atkinson, R. M., Parsons, B. J., and Smyth, D. H., *J. Physiol.*, **135**, 581 (1957).
42. Taylor, W. R., and Langdon, R. G., *Biochim. et Biophys. Acta*, **21**, 384 (1956).
43. Hawkins, J. D., and Wills, E. D., *Biochim. et Biophys. Acta*, **23**, 210 (1957).
44. Chinard, F. P., Taylor, W. R., Nolan, M. F., and Enns, T., *Science*, **125**, 736 (1957).
45. Csáky, T. Z., and Wilson, J. E., *Biochim. et Biophys. Acta*, **22**, 185 (1956).
46. Csáky, T. Z., *Science*, **118**, 253 (1953).
47. Crane, R. K., and Krane, S. M., *Biochim. et Biophys. Acta* **20**, 568 (1956).

β-Hydroxybutyrate and Acetate Metabolism of the Perfused Bovine Udder*

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It was established by Shaw and Knodt (1) that the normal lactating udder (*in situ*) of the cow utilizes almost 2 mg. of β-hydroxybutyric acid per 100 ml. of blood traversing the udder; it was calculated that this was sufficient to account for the short chain fatty acids of milk fat or for about 40 per cent of the oxygen consumption of the secreting gland if it were oxidized for energy purposes. Later work on ketotic cows (2) and perfused udders (3) showed that the utilization of this substrate by the lactating udder is greatly increased when the arterial levels are high. However, the ultimate fate of this substance within the mammary gland tissue remained uncertain.

The importance of the lower aliphatic acids in ruminant metabolism has come to be recognized as a result of current interest in the metabolic activities of rumen microorganisms. In ruminants, acetate, propionate and butyrate are primary products of carbohydrate digestion and very little glucose, as such, is absorbed from the gut. The diurnal variations in the arterial levels of acetate are appreciable and the utilization of acetate by the udder is dependent on the arterial levels (4). Acetate is primarily incorporated by the perfused lactating ruminant udder into the volatile fatty acids characteristic of the ruminant milk fat (5). Butyrate, when injected intravenously, is incorporated to a lesser extent into milk fat, and participates in a net synthesis of carbohydrate in the ruminant (6, 7).

This is a report on the incorporation of carboxyl-labeled β-hydroxybutyrate into milk components by a perfused udder, and on the effect of acetate on the utilization of β-hydroxybutyrate by the perfused udder. A summary of some of the results of this study has been reported.[1]

EXPERIMENTAL

Lactating bovine mammary glands were isolated and perfused by the procedure described earlier (8, 9). One-half of the udder was dissected out and perfused with heparinized blood, while the other half was used as a control.

The volatile fatty acid content of the blood was determined by steam distillation according to Scarisbrick (10) and calculated as acetate. The β-hydroxybutyrate in the blood was determined by the method of Weichselbaum and Somogyi (11). The volatile fatty acid content of the mammary tissue was measured by rendering the tissue macerate slightly alkaline and extracting it with boiling water. The extract was analyzed by the Scarisbrick procedure. It was our experience that the blood samples for acetate determination could not be kept beyond 6 hours, even in ice; the use of perservatives, such as thymol or fluoride, interfered with the analysis.

The utilization of acetate by the perfused lactating mammary gland was first studied by adding known amounts of acetate to the perfusate, initially, and determining blood and tissue acetate at the end of the perfusion. The difference in acetate content between the perfused tissue and the control tissue, if positive, was assumed to be acetate taken up by the gland but not metabolized. Usually, this amount was very small.

In order to study the possible competition between the two substrates, arteriovenous differences, rather than total utilization, were used as the criteria. β-Hydroxybutyrate was added to the blood to give a concentration of 30 to 50 mg./100 ml. Perfusion of the half udder was begun, and one passage of the entire quantity of the blood was allowed in order to bring about equilibrium between blood and tissue. Three sets of arterial and venous samples were drawn simultaneously in the next three successive passages of blood for the determination of the uptake (arteriovenous difference) of β-hydroxybutyrate. Acetate was then added to the blood to obtain a concentration of 10 to 20 mg./100 ml. One complete passage of the blood was allowed for equilibrium and then three sets of arterial and venous samples were drawn for analysis in as many passages of blood. Usually, however, only two sets of samples could be analyzed for acetate since the third set could not be analyzed within the desired 6-hour time period in most cases.

Five perfusions were conducted to form the preliminary work for experiments on possible competition. Since they may be of interest to students of perfusion, the observations made in the course of this work may be summarized without a detailed discussion. Unless an adequate perfusion rate was maintained, equilibrium between blood and tissue was difficult to establish. In perfusions with a good rate of blood flow, one passage of blood sufficed to establish equilibrium. Nonlactating udders showed no uptake of acetate once equilibrium was established. This is similar to observations on the utilization of β-hydroxybutyrate by the perfused udder (3). When acetate was added to the blood, approximately 1 hour after the beginning of perfusion,

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[1] A brief paper presented before the annual meeting of the American Dairy Science Association at Pennsylvania State College, Pennsylvania, 1954.

acetate was utilized in essentially a normal manner, the arteriovenous difference being usually 5 to 6 mg./100 ml. of blood per passage.

Tracer Perfusion—The lactating mammary gland of a cow of known history was perfused in the usual manner. At the beginning of perfusion, 2.33 gm. of β-hydroxybutyrate labeled with C^{14} in the carboxyl group (racemic mixture synthesized by Tracerlab., Inc., Boston) was added to the blood. The total activity added was 1 mc. and the specific activity in the counting system was 3.5×10^4 c.p.m. per μmole. The gland was perfused for 2 hours and 360 ml. of milk was recovered from the udder at the end of perfusion with the aid of an injection of oxytocin (10 i.u.) in the arterial side of the apparatus. The milk was fractionated essentially according to the procedure of Dimant *et al.* (12). The lactose was recrystallized five times from methanol until constant specific activity was reached.

Fractionation of Milk Fat for Butyric Acid—Approximately 1.0 gm. of the milk fat was used for the C^{14} assay of the individual lower fatty acids. Butyric acid was first separated by the procedure of Keeney (13). The forerun and the butyric acid band were titrated separately, rendered alkaline and evaporated to dryness (7.75 mole per cent butyric acid). Four drops of 20 N H_2SO_4 were added to the sodium butyrate extracted with a mixture (1:1) of ether and petroleum ether. The solvent was removed in a vacuum, and the acid dissolved in 1.0 ml. of chloroform and put through the silica gel column for lower fatty acids according to the procedure of Ramsey and Patterson (14). The butyric acid was purified by repeated fractionation by chromatography until constant specific activity was obtained. The butyric acid from the second and third fractionation was found to have the same specific activity.

Preparation of Higher Volatile Fatty Acids—The sodium salts of the acids other than butyric acid were dissolved in 200 ml. of water to which 100 gm. of magnesium sulfate was added. Two ml. of 20 N H_2SO_4 were then added and the solution was distilled until mass crystallization occurred. The volatile fatty acids (both soluble and insoluble in water) were titrated, rendered alkaline with NaOH, and evaporated. The sodium salts were then dissolved in a small quantity of water, made acidic with sulfuric acid, and extracted from liquid with a mixture (1:1) of ether and petroleum ether. Better yields were obtained by liquid-liquid extraction than by adding anhydrous sodium sulfate to the solution followed by extraction. The solvent was evaporated in a vacuum and the acids were fractionated by the Ramsey-Patterson (14) procedure for higher volatile fatty acids. Seven gm. of silica were employed, a procedure which was found to require 7.2 ml. of 98 per cent methanol, 0.3 ml. of the dye solution, and 4 drops of 1.0 N ammonia for the best separation and recovery. The individual acids were rechromatographed to constant specific activity.

Treatment of Higher Acids—The nonvolatile acids remaining in the residue after distillation were extracted with ether and the the solvent evaporated off on a water bath. The acids were separated into saturated and unsaturated fatty acids by a modification of the Twitchell procedure (15). The higher fatty acids were not further fractionated in view of their low specific activity.

Assay for C^{14}—All assays for radiocarbon were made with a windowless gas flow counter and a standard scaler. The solutions were prepared for counting by transferring aliquots containing not more than 200 μg. of materials into aluminum planchets (1½ inches diameter) and drying under an infrared lamp. While it was assumed that this would give "infinitely thin" samples, usually all planchets were made up so as to contain approximately the same weight of material in each. The fatty acids were counted as the sodium salts, the lactose was counted as such and casein was dissolved in 1.0 N HCl and counted in a plastic planchet.

Rate of Blood Flow—By use of the data of Shaw *et al.* (16) the rates of blood flow through the udder *in vivo* were estimated from the levels of milk production. The actual rates obtained during perfusion are expressed as percentages of the estimated rates *in vivo*.

RESULTS

The data on acetate utilization by the perfused udder are shown in Table I. It will be noted that the utilization of acetate was appreciable and relatively uniform. The volatile fatty acids measured in the tissue after perfusion, though calculated as acetate, consisted of higher acids as well; therefore, the figures in Table I represent the minimal quantity of acetate metabolized.

The rates of blood flow in the four perfusions reported in Table I were relatively low (30 to 50 per cent of the estimated rate *in vivo*), although they compare well with those in the literature. The data are presented to illustrate the relative uniformity of acetate uptake by the gland even at such rates of perfusion.

In the three perfusions in which competition was studied, the rates of blood flow approached that estimated for intact udders

TABLE I

Utilization of acetate by perfused bovine udders

Perfusion No.	Duration of perfusion	Volume of blood used	Times blood passed through perfused half*	Acetate							
				In blood		Uptake by perfused half	Final content		Uptake of perfused half		Utilized
				Initial	Final		Perfused half	Control half	Not metabolized	Metabolized	
	min.	*liters*		*mg./100 ml.*		*mg./100 gm. of tissue*				*mg./kg. tissue/min.*	*mg./100 gm. tissue/100 ml. blood*
6	120	6	3	33.8	12.0	31.2				2.6	0.17
7	95	6	6.8	54.9	0.7	31.6	4.1	9.8	−5.7	3.9	0.09
9	50	3	5.4	62.8	13.9	23.8	12.2	6.4	5.8	3.6	0.10
10	120	7	5.9	37.4	15.0	53.3	16.8	13.7	3.1	5.5	0.16

* Calculated from blood flow rates.

TABLE IIA

Summary of three udder perfusions indicating competition between acetate and β-hydroxybutyrate

Perfusion No.	Mean rate of blood flow as percentage of estimated rate *in vivo*	Mean uptake of β-hydroxybutyrate		Mean acetate uptake
		Before adding acetate	After adding acetate	
	%	*mg./100 ml. blood*		
76	75	5.2 (3)*	1.4 (3)	5.8 (2)
80	80	6.2 (3)	0.5 (2)	5.5 (2)
82	110	3.2 (3)	0.8 (1)	5.6 (1)
Mean:		4.8	1.0	5.6
Standard deviation of the mean:		±0.25	±0.15	±0.12

* Numbers in parentheses refer to number of arteriovenous differences.

TABLE IIB

Effect of addition of acetate on utilization of β-hydroxybutyric acid by perfused udder (perfusion 82)

Perfusion time	Rate of blood flow	β-Hydroxybutyric acid in blood			Acetic acid in blood		
		Arterial	Venous	Difference	Arterial	Venous	Difference
min.	*ml./min.*	*mg./100 ml.*			*mg./100 ml.*		
0							
3	800						
23	950	56.7	54.3	2.4	*		
38	1060	53.4	49.8	3.6	*		
50	1100	47.4	43.9	3.5	*		
65	1200	41.5	40.7	0.8	10.0†	4.3	5.7
71	1300	42.2	37.2	5.0	1.2		

* Arterial acetate was too low to be measured (less than 0.05 mg./100 ml.).

† 2 gm. of sodium acetate were added at 52 minutes perfusion time.

TABLE III

Specific activities of milk components isolated from milk recovered after perfusion of isolated bovine udder with 1 mc. of $CH_3 \cdot CHOH \cdot CH_2C^{14}OOH$ in the blood

Component	Specific activity
Precursor in blood	315×10^4 c.p.m./μmole
Milk fat	501 c.p.m./mg.
Casein	399 c.p.m./mg.
Lactose	52 c.p.m./μmole
Total VSFA* from milk fat	770 c.p.m./μmole
Butyric acid	590 c.p.m./μmole
Caproic acid	1118 c.p.m./μmole
Caprylic acid	1396 c.p.m./μmole
Capric acid†	1226 c.p.m./μmole
Lauric acid†	19 c.p.m./μmole
Longer chain acids	Negligible

* Volatile soluble fatty acids.

† Volatile insoluble fatty acids.

in situ. The data obtained from these three perfusions are summarized in Table IIA and the data for one of the perfusions (No. 82) is given in somewhat greater detail in Table IIB.

It will be seen that the uptake of β-hydroxybutyrate was reduced markedly during uptake of acetate. The arteriovenous differences for acetate compare well with those found for live cows (4). The values for β-hydroxybutyrate uptake before addition of acetate are quite similar to those recorded at about the same arterial levels of β-hydroxybutyrate in live cows (1). The possibility of abnormal metabolism of acetate and β-hydroxybutyrate by these perfused udders, therefore, appears remote.

The specific activities of the various components of milk recovered from the perfusion in which carboxyl-labeled β-hydroxybutyrate was used are found in Table III. It was found that there was an appreciable incorporation of label into milk fat and casein but little activity was observed in lactose. The incorporation of the label into the short chain fatty acids was especially high; the specific activity of caproic acid (c.p.m. per μmole) was almost twice that of butyric acid. The specific activity levelled off in the C_8 and C_{10} acids. Negligible incorporation was found in the C_{12} and higher acids.

DISCUSSION

The presence of acetate depresses the uptake of β-hydroxybutyrate by the perfused bovine udder (Tables IIA and IIB). The limitations and the advantages of the perfusion technique are too well recognized to merit detailed discussion here. A study of the present type is not readily conducted without the aid of the perfusion but the application of these findings to the behavior of the udder *in situ* in the live cow must be made with the proper reservations.

β-Hydroxybutyrate is present in the normal bovine arterial blood to the extent of 3 to 6 mg./100 ml. Normally there is no marked diurnal variation in the blood levels of β-hydroxybutyrate (1), whereas arterial acetate levels exhibit diurnal tides of considerable magnitude (4). The competition between acetate and β-hydroxybutyrate may be a mechanism whereby acetate may be utilized efficiently in spite of the large diurnal tides in the blood. Butyrate appears to participate in the net synthesis of carbohydrate in the ruminant by pathways not involving acetate (6, 7); hence an additional advantage of such competition might be in the sparing of the glucose precursors in an animal that has no direct dietary source of glucose. The low specific activity of lactose (Table III) indicates that there is no appreciable carbohydrate synthesis from four carbon intermediates in the isolated udder or that the carboxyl group is lost during the synthesis.

Since glucose is the precursor of lactose, the differences between the data recorded herein and those reported by Kleiber *et al.* (6) and McCarthy *et al.* (7) can best be explained on the basis that C_4 intermediates are converted to carbohydrate elsewhere.

The data are consistent with the beliefs that the substrate was reduced directly to butyrate and that the C_2 pool in the perfused udder is negligible in size (5). The C_2 moieties of β-hydroxybutyrate could not have been utilized identically; a preferential utilization of the carboxyl end of the molecule for lipogenesis (17) is the possible explanation for the higher specific activities of the C_6, C_8, and C_{10} compared to the C_4 volatile fatty acids of the milk fat. Lauric and higher acids were negligibly labeled;

this is in contrast with the work of Cowie *et al.* (5) on acetate, where appreciable labeling was found up to and including palmitic acid. Popják *et al.* (18) suggested, on the basis of earlier studies by Shaw and Knodt (1), that β-hydroxybutyrate was perhaps the C_4 precursor for the volatile fatty acids of milk which was even more important than acetate. The work of Kleiber *et al.* (6) and McCarthy *et al.* (7) would preclude butyrate as such from being this precursor; it is now certain that this precursor is β-hydroxybutyrate.

The diminution in the molar percentage of short chain fatty acids of low ruminant milk fat during fasting (15) or during ketosis (16) is not attributable simply to the absence of dietary acetate and the change takes place in the presence of high levels of β-hydroxybutyrate in blood. It seems logical to assume that mammary lipogenesis from C_2 fragments is inhibited by fasting, by a mechanism similar to that operating in the inhibition of hepatic lipogenesis (19) by fasting. This assumption is strengthened by the fact, now well recognized, that the molar percentage of butyrate in bovine milk fat is not reduced as markedly or as quickly by fasting as the molar percentage of caproic, caprylic, and capric acids (15), indicating that, while mammary lipogenesis from C_2 units is inhibited, direct reduction of β-hydroxybutyrate can maintain butyrate levels relatively high in the milk fat. This is further strengthened by the observation by Keeney (13) that the molar percentage of butyrate in commercial butter is relatively uniform in spite of the wide fluctuations in the Reichert-Meissl value (which is an arbitrary measure of the C_4 and C_6 fatty acids) and the Polenske value (which is an arbitrary measure of the volatile insoluble C_8, C_{10}, and C_{12} volatile fatty acids).

SUMMARY

The carboxyl carbon of C^{14}-labeled β-hydroxybutyrate was incorporated appreciably into milk fat and casein by the perfused lactating bovine udder. Negligible incorporation was found in lactose. The volatile fatty acids of milk fat were labeled in such a manner as to indicate an asymmetrical utilization of the substrate, the carboxyl end being preferentially utilized.

The uptake of β-hydroxybutyrate from the blood by the perfused lactating bovine udder was shown to be depressed by the presence of acetate; acetate was taken up preferentially.

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REFERENCES

1. Shaw, J. C., and Knodt, C. B., *J. Biol. Chem.*, **138**, 287 (1941).
2. Shaw, J. C., *J. Biol. Chem.*, **142**, 53 (1942).
3. Shaw, J. C., and Petersen, W. E., *J. Biol. Chem.*, **147**, 639 (1943).
4. McClymont, G. L., *Australian J. Agri. Research*, **2**, 158 (1951).
5. Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. J., and Popják, G., *Biochem. J.*, **49**, 610 (1951).
6. Kleiber, M., Black, A. L., Brown, M. A., Luick, J., Baxter, C. F., and Tolbert, B. M., *J. Biol. Chem.*, **210**, 239 (1954).
7. McCarthy, R., Shaw, J. C., and Lakshmanan, S., *Proc. Soc. Exptl. Biol. Med.*, **99**, 560 (1958).
8. Petersen, W. E., Shaw, J. C., and Visscher, M. B., *J. Dairy Sci.*, **24**, 139 (1941).
9. Kumar, S., Lakshmanan, S., Corbin, E. A., and Shaw, J. C., *Am. J. Physiol.*, **173**, 83 (1953).
10. Scarisbrick, R., *Biochem. J.*, **50**, xxxiv (1952).
11. Weichselbaum, R. E., and Somogyi, M., *J. Biol. Chem.*, **140**, 5 (1941).
12. Dimant, E., Smith, V. R., and Lardy, H. A., *J. Biol. Chem.*, **201**, 85 (1953).
13. Keeney, M., *J. Assoc. Offic. Agri. Chemists*, **39**, 212 (1956).
14. Ramsey, L. L., and Patterson, W. I., *J. Assoc. Offic. Agri. Chemists*, **28**, 644 (1945).
15. Hilditch, T. P., *The chemical constitution of natural fats*, 2nd edition, Chapman and Hall, Ltd., London, 1947.
16. Shaw, J. C., Powell, R. C., and Knodt, C. B., *J. Dairy Sci.*, **25**, 909 (1942).
17. Chaikoff, I. L., and Brown, G. W., in D. M. Greenberg (Editor), *Chemical pathways of metabolism, Vol. I.*, Academic Press, Inc., New York, 1954.
18. Popják, G., French, T. H., Hunter, G. D., and Martin, A. J. P., *Biochem. J.*, **48**, 612 (1951).
19. Lyon, I., Masri, M. S., and Chaikoff, I. L., *J. Biol. Chem.*, **196**, 15 (1952).

A Sensitive and Stereospecific Enzymatic Assay for Xylulose

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The fundamental investigations of Touster *et al.* (1) and Hollmann and Touster (2) have demonstrated that L-xylulose is converted to D-xylulose by two pyridine nucleotide linked dehydrogenases active on a common substrate, xylitol. The reactions may be written as follows:

$$\text{L-Xylulose} + \text{TPNH} + \text{H}^+ \rightleftarrows \text{xylitol} + \text{TPN}^+ \quad (1)$$

$$\text{Xylitol} + \text{DPN}^+ \rightleftarrows \text{D-xylulose} + \text{DPNH} + \text{H}^+ \quad (2)$$

In an attempt to utilize these reactions for the quantitative determination of the stereoisomers of xylulose, the original method of preparation of Hollmann and Touster (2), which involved the butanol solubilization of guinea pig mitochondria, was investigated. In our hands, the enzymes so obtained could not be used for assay purposes due to their apparently low substrate affinity. Further investigation revealed that a water extract of guinea pig liver acetone powder yielded a high potency preparation which contained both enzymes and was suitable for analytical use. The ready resolution and purification of these enzymes permitted the development of a rapid, sensitive assay capable of measuring either L- or D-xylulose alone or in mixtures.

EXPERIMENTAL

Materials—D-Xylulose was prepared by the action of *Acetobacter suboxydans* on D-arabitol (3). The arabitol was a gift of Dr. H. B. Wood of this institute. L-Xylulose and D- and L-ribulose were prepared as described previously (4). Sedoheptulose was generated from the crystalline hexaacetate provided by Dr. N. Richtmeyer, by treatment with sodium methoxide. L-Erythrulose was a gift of Dr. B. L. Horecker. Alumina gel Cγ was prepared as described by Willstätter *et al.* (5) and calcium phosphate gel by the method of Keilin and Hartree (6). All other substrates and cofactors were obtained from commercial sources.

Analytical Procedures—Free ketopentose concentrations were measured by the cysteine carbazole method (7) as described previously (8). Protein was determined by the optical method of Warburg and Christian (9) or by the phenol method of Lowry *et al.* (10).

Enzyme activities were followed by observing the rate of oxidation of reduced pyridine nucleotides at 340 mμ in the Beckman DU Spectrophotometer with the use of D- or L-xylulose as substrate. A unit is defined as that amount of enzyme which will catalyze an optical density decrease of 1.0 per minute under the conditions of the assay. Specific activity is defined as the number of units per mg. of protein (Fig. 1).

* Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry at Georgetown University, Washington, D. C.

Purification of TPN-Xylitol (L-Xylulose) Dehydrogenase

Extraction—Guinea pig liver acetone powder, 5 gm., was suspended in 100 ml. of distilled water, stirred for 10 minutes at room temperature, and centrifuged. The clear, dark-colored supernatant solution (Fraction 1) which contains both the TPN- and DPN-linked dehydrogenases, is not stable and must be carried rapidly through the ammonium sulfate step before the enzyme can be stored.

Fractionation—The extract was heated rapidly to 50° in a boiling water bath and then transferred to a constant temperature bath at 50° for 5 minutes. The solution was then chilled in ice to room temperature and adjusted to pH 5.8 with 1.0 N acetic acid. The precipitate was removed by centrifugation and rejected; the supernatant fluid represents Fraction 2.

Fraction 2, which contained 575 mg. of protein was treated with an equal amount (575 mg. dry weight) of calcium phosphate gel. The purification obtained was by absorption of inactive material and the gel suspension was centrifuged to minimize volume increases. Thus 96 ml. of the gel preparation containing 6 mg. of calcium phosphate gel per ml. were centrifuged and the gel suspended directly in the enzyme solution. After standing 10 minutes at 0° the preparation was centrifuged and the gel discarded (Fraction 3).

The clear, straw-colored supernatant fluid was treated with an equal volume of a cold (2°), saturated ammonium sulfate solution, which had been previously adjusted to pH 8.0 with concentrated NH_4OH. After standing at 0° for approximately 15 minutes the precipitate, containing the TPN-dehydrogenase, was collected by centrifugation and dissolved in a small volume of water (Fraction 4).

The supernatant solution (Fraction 5) (0.5 of saturation) containing the DPN-dehydrogenase can be saved for the preparation of this enzyme if so desired. This solution was found to be stable for several weeks at 0°.

Fraction 4, 5 ml., was chilled in an alcohol-ice mixture to 0° and 5.0 ml. of cold acetone (−14°) were slowly added. During the course of the addition the temperature of the mixture was gradually lowered to −8°. The precipitate, containing approximately 20 per cent of the total activity was removed by centrifugation and rejected. A second 5 ml. of acetone were added to the enzyme as above and the precipitate collected and dissolved in 2 ml. of water (Fraction 6). This solution constitutes a highly active preparation of the TPN-dehydrogenase of approximately 100-fold purification and is completely free of DPN-dehydrogenase activity. A summary of the purification procedure is given in Table I.

Purification of DPN-Xylitol (D-Xylulose) Dehydrogenase—To 178 ml. of Fraction 5 described above were added 16.4 gm. of

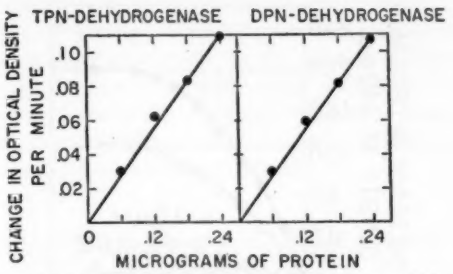

FIG. 1. Relationship between the reaction rate and the enzyme concentration with the use of a crude extract. The reaction mixture contained 40 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.0, 1.0 μmole of cysteine, 0.10 μmole of TPNH or DPNH, 5.0 μmoles of $MgCl_2$ and 1.0 μmole of D- or L-xylulose in a total volume of 1.0 ml.

TABLE I

Summary of enzyme purification

Fraction No.	Treatment	Volume	Total protein	TPN-dehydrogenase		DPN-dehydrogenase	
				Total units	Specific activity	Total units	Specific activity
		ml.	*mg.*		*units/mg.*		*unit/mg.*
1	Crude extract	89	1080	600	0.56	555	0.51
2	pH precipitation	84	575	400	0.70	436	0.76
3	Calcium phosphate	89	190	460	2.4	840	4.4
4	Ammonium sulfate (0–0.5 saturation)	5.0	76.5	390	5.1	0	0
6	Acetone	2.0	2.36	150	63	0	0
7	Ammonium sulfate (0.5–0.65 saturation)	5	80	0	0	550	6.9
8	Acetone	2	11.3	0	0	260	23

solid ammonium sulfate to bring the concentration to 65 per cent of saturation. After standing 15 to 20 minutes at 0° the precipitate was collected by centrifugation and dissolved in a small amount of water (Fraction 7).

To 5.0 ml. of Fraction 7, chilled to −2° were added 2.5 ml. of cold acetone (−14°), the temperature of the mixture being gradually lowered to −8° during the course of the addition. The solution was centrifuged at 0° and the precipitate was rejected. Another 2.5 ml. of cold acetone were added to the supernatant fluid and the precipitate was collected and dissolved in 2 ml. of water. This preparation, representing a 40-fold purification, was stable at −10° for several weeks if repeated freezing and thawing were avoided.

Properties of Enzymes

Stability—Both the TPN- and DPN-linked dehydrogenases are unstable in the crude state and preparations should be brought to the ammonium sulfate step to avoid extensive destruction of the enzymes. After ammonium sulfate fractionation the preparations may be stored at ice box temperatures for several days without noticeable loss of activity.

Effect of pH—The reaction with either enzyme proceeds with maximal velocity at pH 7.0 (Fig. 2). The rates of reaction declined sharply on either side of the maximum.

Inhibitors and Activators—The partially purified enzymes require cysteine (10^{-3} M) for optimal activity. The TPN- and DPN-dehydrogenases were inhibited completely by *p*-chloromercuribenzoate at 10^{-3} M. Neither enzyme was inhibited by Versene (ethylenediaminetetraacetate), or iodoacetate at 10^{-3} M although an iodoacetate inhibition of the DPN-xylitol dehydrogenase in guinea pig liver has been reported by Hollmann and Touster (2). No evidence for a metal cofactor was obtained.

Substrate Specificity—The TPN-dehydrogenase appears to be specific for L-xylulose. The DPN-dehydrogenase is less specific, in that the most highly purified preparations utilized L-erythrulose at the same rate as D-xylulose and had minimal activity with L-xylulose, D-fructose, and L-ribulose (Table II). The TPN dehydrogenase was inactive when DPNH was substituted for TPNH; the DPN enzyme exhibited a corresponding specificity.

Effect of Substrate Concentration—The rate of oxidation as a function of L-xylulose concentration with the TPN-enzyme is given in Fig. 3. The K_m calculated from a Lineweaver-Burk plot (11) was 2.9×10^{-4} M. With the DPN-enzyme, the K_m was 6.6×10^{-4} M (Fig. 4).

Maximal velocity was obtained at a D-xylulose concentration of 1×10^{-3} M. Substrate concentrations in excess of this value

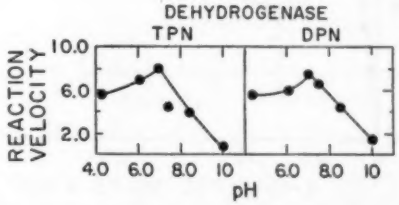

FIG. 2. Variation of xylitol dehydrogenase activities with pH. Assay conditions are as described in Fig. 1. TPN enzyme, 16 μg., (calcium phosphate gel, specific activity = 24) and 11 μg. of DPN enzyme (calcium phosphate gel, specific activity = 4.4) were used. All determinations were made with phosphate buffers.

TABLE II

Substrate specificity

The reaction mixture contained 40 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.0; 1.0 μmole cysteine; 0.10 μmole TPNH or DPNH; 5.0 μmoles $MgCl_2$; 0.09 unit of enzyme; and 1.0 μmole of the respective substrate in a total volume of 1.0 ml. The specific activity of the TPN-dehydrogenase was 63; that of the DPN-dehydrogenase, 23 (Table I).

Substrate	Maximum activity	
	TPN-dehydrogenase	DPN-dehydrogenase
	%	%
L-Xylulose	100.0	3.9
D-Xylulose	0.0	100.0
D-Fructose	0.0	4.2
L-Ribulose	6.5	3.0
D-Ribulose	0.0	0.0
D-Ribose	0.0	0.0
D-Xylose	0.0	0.0
L-Xylose	0.0	0.0
L-Sorbose	0.0	1.7
D-Altroheptulose	0.0	0.9
L-Erythrulose	0.0	109.0
D-Galacturonic acid	0.0	0.0

lead to a marked inhibition. All determinations were made with pyridine nucleotide concentration of 1×10^{-4} M.

Stoichiometry—At pH 7.0 and in the presence of an excess of the reduced pyridine nucleotides and the corresponding dehydrogenase, D- and L-xylulose were completely reduced to xylitol. In the range of 0.01 to 0.06 μmole of substrate, stoichiometric amounts of DPNH or TPNH were oxidized (Figs. 5 and 6).

Reversibility—In order to demonstrate the reversibility of these reactions it was necessary to raise the pH of the reaction mixture to 10 and to increase the substrate concentrations 10- to 20-fold.

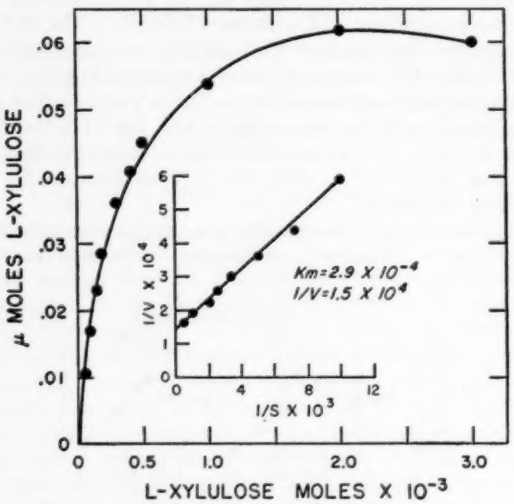

FIG. 3. The effect of L-xylulose concentration on the reaction velocity. The assay conditions were as described in Fig. 1 and contained 1.0 μg. of enzyme (specific activity = 63). Ordinate indicates μmoles reacting in 5 minutes.

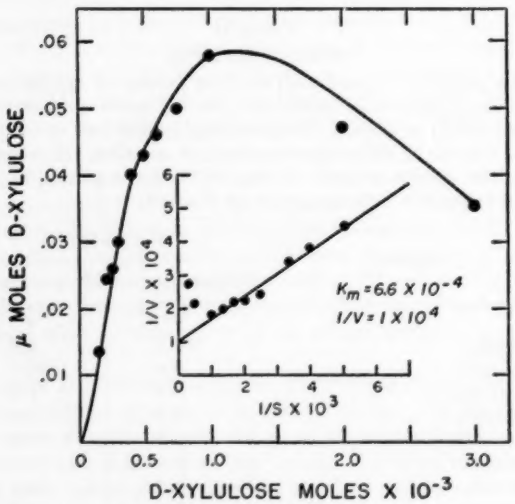

FIG. 4. The effect of D-xylulose concentration on the reaction velocity. The assay conditions were as described in Fig. 1 and contained 2.8 μg. of enzyme (specific activity = 23). Ordinate indicates μmoles of D-xylulose reacting in 5 minutes.

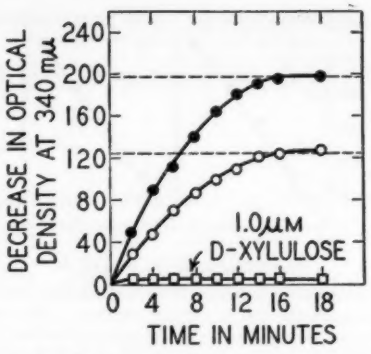

FIG. 5. Stoichiometry of TPN-dehydrogenase reaction. L-Xylulose concentration .02, and .03 μM, respectively. Dotted lines indicate theoretical values. The reaction conditions were as described in Fig. 1 with 1.0 unit of enzyme (specific activity = 60).

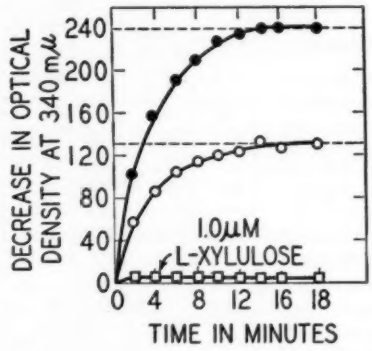

FIG. 6. Stoichiometry of the DPN-dehydrogenase reaction. D-Xylulose concentration .021 μM and .039 μM, respectively. Dotted line indicates theoretical value. The reaction conditions were as described in Fig. 1 with 1.0 unit of enzyme (specific activity = 23).

Significant reduction required the addition of a large excess of enzyme. No activity was obtained with D- and L-arabitol, D-ribitol, D-sorbitol, or L-iditol with the TPN-enzyme. However, under these conditions, activity of the DPN-dehydrogenase could be demonstrated with all the above sugar alcohols except for D-arabitol.

DISCUSSION

The nomenclature of the enzymes involved in the conversion of L-xylulose to D-xylulose has not been definitely established. In agreement with a suggestion from Dr. Touster[1] the following names are proposed: TPN-xylitol (L-xylulose) dehydrogenase, and DPN-xylitol (D-xylulose) dehydrogenase.

Because of the high affinity of the dehydrogenases for their respective substrates these reactions have been found to be suitable for the quantitative determination of extremely small amounts of the respective sugars.

The TPN-linked enzyme appears to be specific for L-xylulose,

[1] Personal communication.

whereas the DPN-dehydrogenase reacts equally well with D-xylulose and L-erythrulose. Since the presence of L-erythrulose can be determined colorimetrically by the method of Dische and Dische (12), this lack of specificity should not interfere with the usefulness of the assay for D-xylulose. Moreover, the fact that L-erythrulose is utilized may prove of value as an analytical tool for the investigation of tetrose metabolism.

SUMMARY

A rapid enzymatic assay method for the determination of D- and L-xylulose alone or in mixtures has been described. The assays depend upon the specificities of two enzymes, TPN-xylitol (L-xylulose) dehydrogenase, and DPN-xylitol (D-xylulose) dehydrogenase. The enzymes have been purified from a guinea pig acetone powder. A study of their properties is reported.

REFERENCES

1. Touster, O., Reynolds, V. H., and Hutcheson, R. N., *J. Biol. Chem.*, **221**, 697 (1956).
2. Hollmann, S., and Touster, O., *J. Biol. Chem.*, **225**, 87 (1957).
3. Hann, R. M., Tilden, E. B., and Hudson, C. S., *J. Am. Chem. Soc.*, **60**, 1201 (1938).
4. Hickman, J., and Ashwell, G., *J. Biol. Chem.*, **232**, 737 (1958).
5. Willstätter, R., Kraut, H., and Erbacher, O., *Ber. deut. chem. Ges.*, **58**, 2448 (1925).
6. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, B.*, **124**, 397 (1938).
7. Dische, Z., *J. Biol. Chem.*, **204**, 983 (1953).
8. Ashwell, G., and Hickman, J., *J. Biol. Chem.*, **226**, 65 (1957).
9. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
11. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
12. Dische, Z., and Dische, M. R., *Biochim. et Biophys. Acta*, **27**, 184 (1958).

Biosynthesis of Glycolate and Related Compounds from Ribose-1-C^{14} in Tobacco Leaves*

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Previous work in this laboratory has shown that the α carbon of glycolate, the α carbon of glycine, and the β carbon of serine are efficient precursors for the methyl groups of nicotine and lignin in tobacco plants (1–3) and of pectin in radish plants (4). However, a metabolic pathway by which methyl groups may be synthesized from carbon dioxide in the photosynthetic process has not been elucidated. Glycolate appears to be a key intermediate in methyl group formation since it is utilized for synthesis of glycine and serine (5, 6) and, after administration of $C^{14}O_2$, it is labeled rapidly in photosynthesis (7, 8). The mechanism of formation of glycolate is not known, although there has been some speculation concerning its origin. Wilson and Calvin (9) have suggested that glycolate is synthesized from the pentose phosphates of the photosynthetic cycle. Recently, Weissbach and Horecker (10) reported that ribose-1-C^{14}-5-phosphate was converted, in an extract from spinach leaf, to glycine labeled predominantly in the α carbon, and presumably glycolate was an intermediate in this reaction.

Because of the possible importance of glycolate, and compounds formed from it, as precursors of methyl groups, it seemed of interest to study the biogenesis of these compounds in detached tobacco leaves.

The present study demonstrated that radioactive glycolate, glycine, serine, and alanine were synthesized from D-ribose-1-C^{14} when the sugar was metabolized by tobacco leaves in light and in the dark. Illumination of leaves during the period of metabolism increased the total amount of C^{14} incorporated into various compounds, but the distribution of C^{14} within these compounds in light and dark metabolism was similar. The C^{14} was incorporated principally into the α carbon atoms of glycolate and glycine, and into the α and β carbons of serine. Alanine was labeled predominantly in the β carbon.

EXPERIMENTAL

Leaves, weighing 0.5 to 0.6 gm., from 3-month-old tobacco plants (*Nicotiana rustica L.*) were used in this study. Samples of D-ribose-1-C^{14} to be fed to the plants were cochromatographed with authentic D-ribose in an aqueous phenol solvent (11).[1] One radioactive area was detected, and the R_F value was the same as that of the authentic sample of D-ribose in each case.

The detached leaves were vacuum infiltrated with an aqueous solution containing 2 mg. of D-ribose-1-C^{14} (8×10^5 c.p.m.) per ml., and each leaf took up about 0.25 ml. of solution. One group of leaves was placed in a dark box and another group in light. The leaves were removed 3 hours after administration of the radioactive ribose, cut into small pieces, and placed in boiling 80 per cent ethanol. The mixture was cooled, disintegrated in a glass homogenizer, and filtered. The filtrate was used for isolation of the amino acids and glycolic acid.

Isolation and Purification of Glycine, Serine, Alanine, and Glycolic Acid—The amino acids were separated from the leaf extracts by one-dimensional paper chromatography. Three solvents were employed in the following order: *tert*-amyl alcohol saturated with acetate buffer, pH 5.6 (12); phenol saturated with an aqueous solution containing 3.7 per cent of sodium dihydrogen phosphate and 6.3 per cent of sodium citrate (11); and pyridine and water (13:7 by volume) (11). The amino acids were eluted from the chromatogram with water, and the eluate was placed on another filter paper sheet for resolution with the succeeding solvent system. After resolution with the third solvent, the amino acids were eluted, from 20 to 100 mg. of carrier amino acid were added to the solution, and the radioactive amino acids were crystallized. The amino acids were then recrystallized to constant specific activity, two recrystallizations usually being sufficient.

Glycolic acid was separated from the leaf extract by paper chromatography. Two solvents were used; the first was composed of chloroform, 95 per cent ethanol, and 90 per cent formic acid (1:1:0.01 by volume) and the second was isooctane, 95 per cent ethanol, acetone, and 90 per cent formic acid (4:4:1:0.01 by volume) (13).

The glycolic acid was eluted with water and recrystallized with carrier calcium glycolate. Calcium glycolate was converted to carbon dioxide by combustion for determination of the specific activity.

Unchanged ribose was estimated by chromatographing the leaf extract with *tert*-amyl alcohol saturated with acetate buffer, pH 5.6, and measuring the radioactivity in a spot corresponding in R_F value with that of authentic D-ribose.

Degradation Procedures—Glycine was degraded by the ninhydrin method (14), and the carbon dioxide was collected as barium carbonate. The formaldehyde formed from the α carbon was collected as the dimedon derivative.

Serine was degraded by the procedure of Bassham *et al.* (15) with modifications suggested by Aronoff (8). The amino acid

* The data were taken from a thesis presented by Thomas Griffith in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Michigan State University. The work was supported by a grant from the National Institutes of Health. This paper was presented in part at the meeting of the American Society of Plant Physiologists in Bloomington, Indiana, August 1958.

[1] We are indebted to Dr. Martin Gibbs for our first sample of ribose-1-C^{14}. The remainder was purchased from Nuclear-Chicago Corporation, Chicago, Illinois.

was oxidized with periodic acid, and the formaldehyde was isolated as the dimedon derivative. The glyoxylic acid remaining was oxidized with 0.5 M cerate in 6 M perchloric acid,[2] and the carbon dioxide was collected as barium carbonate. Calcium glycolate was degraded by the procedure used for glyoxylic acid.

Alanine was degraded (14) with ninhydrin, and the carbon dioxide was isolated as barium carbonate. The remaining acetaldehyde was allowed to react with sodium hypoiodite, and the iodoform arising from the β carbon was converted to carbon dioxide by combustion. The remaining formic acid, which represented the α carbon of the alanine, was oxidized to carbon dioxide with mercuric oxide and was collected as barium carbonate.

Determination of Radioactivity—The radioactivity of the various compounds was determined with a Nuclear-Chicago 192x scaler and a Tracerlab model SC-16 proportion flow counter. The specific activity of all compounds was corrected for self-absorption. Radioactivity on paper chromatograms was measured with a Forro Scientific Company paper strip scanner.

RESULTS AND DISCUSSION

About 20 per cent of the D-ribose-1-C^{14} administered in the light was recovered unchanged from the leaves after the 3-hour metabolism period, whereas 40 per cent was recovered unchanged from the leaves allowed to metabolize in the dark.

About 0.5 per cent of the radioactive carbon administered was incorporated into glycine and glycolate, 1 per cent into serine, and 3 per cent into alanine in the light, whereas, in the dark, alanine and serine each contained about 0.5 per cent and glycolate about 0.1 per cent of the administered radioactivity. Glycine from leaves fed ribose in the dark was not degraded since it contained an insignificant quantity of C^{14}. This result is in agreement with other reports which show little or no synthesis of glycine in the absence of light (7, 16). The distribution of C^{14} in glycine, serine, glycolate, and alanine isolated from leaves which were administered D-ribose-1-C^{14} in the light and in the dark is presented in Table I. Two separate degradations were made of each compound, with the exception of glycolate from leaves fed D-ribose-1-C^{14} in the dark, and the values reported are the averages of these two analyses. The duplicate analyses agreed within 10 per cent.

The location of C^{14} within the molecules was similar in both light and dark experiments. Glycolate and glycine had essentially the same distribution of C^{14} in the carboxyl and α carbons, and serine had a similar distribution in these carbons. These results confirm the known interconversions of these three compounds (5, 6), and indicate that they arise in large part from a common two-carbon precursor (17, 18). In animal metabolism, serine has also been shown to arise from a three-carbon precursor, 3-phosphoglyceric acid (19). In addition, it has been suggested that serine may be synthesized in plants by a similar mechanism, or by a direct conversion of alanine to serine (14, 20). The distribution of C^{14} in alanine and serine was not similar, and it may therefore be concluded that at best only a small proportion of serine was synthesized directly from alanine.

Serine contained slightly more C^{14} in the β carbon than in the α carbon. Tolbert and Cohan (5) fed plants glycolic acid-2-C^{14} and isolated serine labeled about equally in the α and β carbons.

[2] The cerate was purchased from the G. Frederick Smith Chemical Company, Columbus, Ohio.

TABLE I

Distribution of radioactivity in several products after metabolism of ribose-1-C^{14} in detached tobacco leaves in light and in dark

Compound	Per cent of total radioactivity in each carbon of molecule					
	Carboxyl carbon		α-Carbon		β-Carbon	
	Light	Dark	Light	Dark	Light	Dark
Glycolate	36	31	74	65		
Glycine	29		68			
Serine	11	6	38	38	44	52
Alanine	14	24	9	7	63	62

In their experiments, the carbon chain of glycolate was incorporated unchanged into the carboxyl and α carbon of serine. The β carbon of serine was synthesized from the α carbon of glycolate with little or no dilution, and thus it appeared that the one-carbon pool was small.

In order to account for the higher specific activity of the β carbon of serine in the present experiments, one can assume that the one-carbon pool, synthesized from the α carbon of glycolate, also was small as compared to the glycine pool.

Wilson and Calvin (9) have suggested that glycolate is synthesized in the photosynthetic carbon cycle by a cleavage of a glycolyl-enzyme complex, an intermediate in transketolase reactions. Thus, the carbon chain of glycolate would be synthesized from carbon atoms 1 and 2 of pentose phosphates. If no randomization of C^{14} occurred when D-ribose-1-C^{14} was administered to leaves, the glycolate produced by this mechanism would be labeled only in the α carbon. The observed labeling pattern shows substantial incorporation of C^{14} into the carboxyl carbon. This distribution of C^{14} may be explained by known enzymatic reactions. Horecker *et al.* (21) have demonstrated the conversion of ribose-1-C^{14} 5-phosphate to hexose phosphate labeled in carbon atoms 1 and 3 in a rat liver preparation. The enzymes necessary for these reactions have been shown to occur in plant tissues (22). Oxidation of hexose phosphate through the pentose shunt would yield pentose phosphates labeled in carbon 2. Thus, both pentose phosphate-1-C^{14} and -2-C^{14} would be present. Synthesis of a two-carbon compound from these pentose phosphates would result in glycolate which would be labeled in the α carbon and, to a lesser extent, in the carboxyl carbon.

The synthesis of glyoxylate by way of the glyoxylate cycle (23) followed by utilization of the glyoxylate as a precursor of glycolate, glycine, and serine is another possible metabolic pathway. However, Carpenter and Beevers (24) have reported that the enzymes of the glyoxylate cycle could be demonstrated only in certain plant tissues which had a high lipide content and could not be detected in the tobacco plant. It therefore appears unlikely that glycolate is produced from ribose by way of the glyoxylate cycle in these studies.

The results of the present experiments show that glycolate, glycine, and serine were synthesized from a two-carbon precursor which probably arose from carbon atoms 1 and 2 of a pentose in the photosynthetic carbon cycle. Since it was previously demonstrated that these compounds contribute to the one-carbon pool in plant metabolism, a pathway by which methyl groups can be synthesized from photosynthetic carbon dioxide is therefore established.

SUMMARY

1. Vacuum infiltration of *Nicotiana rustica* leaves with D-ribose-1-C^{14} resulted in the synthesis of radioactive glycolic acid, glycine, serine, and alanine.

2. Illumination of leaves during the period of metabolism increased the total amount of C^{14} incorporated into these compounds, but the distribution of C^{14} within the compounds was similar in light and dark metabolism.

3. The C^{14} was incorporated principally into the α carbons of glycolate and glycine, the α and β carbons of serine, and the β carbon of alanine. The pattern of labeling in these compounds was taken as evidence that carbon 1 and 2 of pentoses gave rise to glycolate which was then converted to glycine and serine.

4. The labeling of alanine after administration of ribose-1-C^{14} indicated that serine was synthesized mainly by a one-carbon plus two-carbon condensation and only to a small extent from alanine or a related three-carbon compound.

REFERENCES

1. Byerrum, R. U., Hamill, R. L., and Ball, C. D., *J. Biol. Chem.*, **210**, 645 (1954).
2. Byerrum, R. U., Ringler, R. L., Hamill, R. L., and Ball, C. D., *J. Biol. Chem.*, **216**, 371 (1955).
3. Byerrum, R. U., Dewey, L. J., Hamill, R. L., and Ball, C. D., *J. Biol. Chem.*, **219**, 345 (1956).
4. Wu, P.-H. L., and Byerrum, R. U., *Plant Physiol.*, **33**, 230 (1958).
5. Tolbert, N. E., and Cohan, M. S., *J. Biol. Chem.*, **204**, 649 (1953).
6. Schou, L., Benson, A. A., Bassham, J. A., and Calvin, M., *Physiol. Plantarum*, **3**, 487 (1950).
7. Benson, A. A., and Calvin, M., *J. Exptl. Botany*, **1**, 63 (1950).
8. Aronoff, S., *Arch. Biochem. Biophys.*, **32**, 237 (1951).
9. Wilson, A. T., and Calvin, M., *J. Am. Chem. Soc.*, **77**, 5948 (1955).
10. Weissbach, A., and Horecker, B. L., in W. D. McElroy and H. B. Glass (Editors), *A symposium on amino acid metabolism*, Johns Hopkins Press, Baltimore, 1955, p. 741.
11. Berry, H. K., Sutton, H. E., Cain, L., and Berry, J. S., *University of Texas publication 5109*, University of Texas, Austin, 1951, p. 22.
12. Tso, T. C., and Jeffrey, R. N., *Arch. Biochem. Biophys.*, **43**, 269 (1953).
13. Stark, J. B., Goodban, A. E., and Owens, H. S., *Anal. Chem.*, **23**, 413 (1951).
14. Vernon, L. P., and Aronoff, S., *Arch. Biochem.*, **29**, 179 (1950).
15. Bassham, J. A., Benson, A. A., and Calvin, M. J., *J. Biol. Chem.*, **185**, 781 (1950).
16. Tolbert, N. E., *J. Biol. Chem.*, **215**, 27 (1955).
17. Tolbert, N. E., and Gailey, F. B., *Plant Physiol.*, **30**, 491 (1955).
18. Rabson, R., and Tolbert, N. E., *Plant Physiol.*, Suppl. 32, xxxv (1957).
19. Ichihara, A., and Greenberg, D. M., *J. Biol. Chem.*, **224**, 331 (1957).
20. Saltman, P., Lynch, V. H., Kunitake, G. M., Stitt, C., and Spolter, H., *Plant Physiol.*, **32**, 197 (1957).
21. Horecker, B. L., Gibbs, M., Klenow, H., and Smyrniotis, P. Z., *J. Biol. Chem.*, **207**, 393 (1954).
22. Axelrod, B., Bandurski, R. S., Greiner, C. M., and Jang, R., *J. Biol. Chem.*, **202**, 619 (1953).
23. Kornberg, H. L., and Krebs, H. A., *Nature*, **179**, 988 (1957).
24. Carpenter, W., and Beevers, H., *Plant Physiol.*, Suppl. 33, xxxv (1958).

Propionic Acid Metabolism

II. ENZYMATIC SYNTHESIS OF LACTYL PANTETHEINE

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The study of propionate metabolism in a number of systems (1-8) has revealed that propionate is initially activated to propionyl coenzyme A and then dehydrogenated to acrylyl coenzyme A. Subsequent metabolism of acrylyl-CoA has been shown to lead to either β-alanyl-CoA, as recently reported by this laboratory (6), or β-hydroxypropionyl-CoA (7, 8).

A search for other enzyme systems capable of catalyzing the decomposition of acrylyl pantetheine, which has been used in place of acrylyl-CoA (1, 6), has revealed a new pathway. Extracts of a microorganism, *Pseudomonas* sp. (isolated from an aerobic enrichment culture[1] containing propionic acid as the only source of carbon and hydrogen) and of pigeon heart muscle, contain an enzyme that catalyzes the hydration of acrylyl pantetheine to form lactyl pantetheine:

$$CH_2{=}CHCOSPa + H_2O \rightarrow CH_3CHOHCOSPa \quad (1)$$

This report describes the new enzyme, acrylyl-CoA hydrase, and presents evidence that the product of the reaction is lactyl pantetheine. An enzyme that catalyzes the deacylation of lactyl-CoA is also described.

METHODS

Chromatographic comparison of hydroxamic acid derivatives of thiolesters was used extensively in the studies described below. Thiolesters were converted to their hydroxamic acids and prepared for chromatography as described previously (9). Table I presents the R_F values, in two solvent systems, of the major compounds that were studied.

The characteristic alkali lability of most thiolesters (10) was made use of in two ways. First, the amount of a saturated thiolester present could be assayed by noting the decrease in ultraviolet absorbance at 232 mμ caused by hydrolysis. It was assumed that the extinction coefficient of lactyl pantetheine is the same as that of lactyl-*N*-acetylcysteamine (11). Lactyl thiolesters were hydrolyzed in 0.5 N potassium hydroxide for 30 minutes at room temperature. Second, the products of the hydrolyzed thiolester could be studied and identified. Lactic dehydrogenase was utilized in identification of the lactate from the hydrolyzed lactyl pantetheine. The equilibrium of the lactic dehydrogenase reaction strongly favors lactate (12). A mechanism used to force the complete oxidation of lactate was the coupling of the lactic dehydrogenase system to 2,3,5-triphenyltetrazolium chloride (TTZ) as a terminal electron acceptor (with a diaphorase as an electron carrying intermediary). 2,3,5-Triphenyltetrazolium chloride has the advantage of being colorless and soluble in the oxidized form, whereas it is red and insoluble when reduced to a formazan (13). The reactions can be visualized as follows:

$$\text{Lactate} + \text{DPN}^+ \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{pyruvate} + \text{DPNH} + \text{H}^+ \quad (2)$$

$$\text{DPNH} + \text{H}^+ + \text{flavin} \xrightarrow{\text{diaphorase}} \text{flavin } H_2 + \text{DPN}^+ \quad (3)$$

$$\text{TTZ} + \text{flavin } H_2 \rightarrow \text{formazan} + \text{flavin} \quad (4)$$

$$\text{Sum: lactate} + \text{TTZ} \rightarrow \text{pyruvate} + \text{formazan} \quad (5)$$

Experimental conditions were adjusted so that the amount of 2,3,5-triphenyltetrazolium chloride reduced was proportional to the quantity of lactate added. There was a straight line relationship between lactate added (0.025 to 0.15 μmole) and the intensity of color produced (Fig. 1). No color was produced when lactic dehydrogenase was omitted. Substitution of β-hydroxypropionate for lactate led to no formazan formation. This system provided a convenient quantitative assay for lactate which clearly differentiated it from β-hydroxypropionate.

The calcium salt of L(+)-lactic acid (California Foundation for Biochemical Research) was converted to the free acid by passing it over a small Dowex 50 column in the hydrogen form. Lactyl thiolesters[2] of both pantetheine and CoA were prepared by the method of Wieland and Köppe (14). Lactyl pantetheine was purified by paper chromatography (*n*-butanol-water, 100:18); lactyl-CoA was chromatographed in a different solvent system (equal volumes of ethanol and 0.1 N sodium acetate, pH 4.5). Both compounds gave single hydroxamate spots on chromatography in solvents A and B (Table I), had ultraviolet absorption maxima at 232 mμ, and yielded equivalent amounts of lactate and mercaptan after alkaline hydrolysis.

Lactyl pantetheine was made enzymatically with the *Pseudomonas* sp. enzyme. Incubation mixtures contained 50 μmoles of triethanolamine hydrochloride buffer, pH 7.5, 5.0 μmoles of acrylyl pantetheine, 1.4 mg. of enzyme protein, and water to a total volume of 0.84 ml. A blank contained no acrylyl pantetheine. Samples were incubated at 30°. Aliquots were examined spectrophotometrically for decrease in absorbance at

[1] This organism was isolated from soil enrichment cultures by Dr. W. B. Jakoby.

[2] We are indebted to Dr. H. T. Miles for assistance and advice in the synthesis of the lactyl thiolesters.

TABLE I

Hydroxamic acid derivatives of thiolesters

To 2.0 μmoles of each of the thiolesters was added 1.0 mmole of neutral hydroxylamine. In each case, half of the resultant hydroxamic acid was chromatographed in Solvent A (secondary butanol-formic acid-water, 75:13:12) and half in Solvent B (*n*-butanol, water, 100:18).

Thiolester	R_F of hydroxamic acid	
	Solvent A	Solvent B
Acrylyl pantetheine	0.20	0.15
Lactyl pantetheine	0.55	0.48
β-Alanyl pantetheine	0.13	0.08

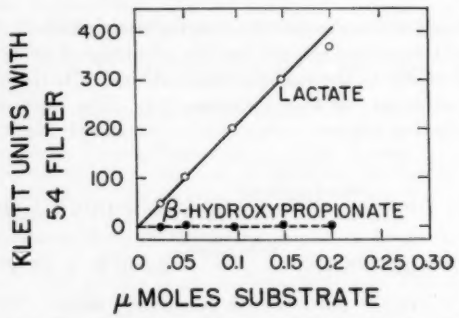

FIG. 1. Assay of lactate with 2,3,5-triphenyltetrazolium chloride. Each tube contained 50 μmoles of potassium phosphate buffer, pH 7.0; 0.05 ml. of 2,3,5-triphenyltetrazolium chloride solution, 50 mg. per ml.; potassium lactate or potassium β-hydroxypropionate as indicated; 4.0 μmoles of DPN; 1.0 unit of lactic dehydrogenase; 1.5 mg. of protein of the diaphorase preparation; and water in a total volume of 0.5 ml. The tubes were gassed with helium for 2 minutes before the addition of diaphorase and then stoppered and incubated at 30° for 12 minutes. Ethanol, 2.2 ml., was added to stop the reactions, dissolve the reduced 2,3,5-triphenyltetrazolium chloride (formazan), and precipitate the protein. After removal of the precipitate by centrifugation, the supernatant solutions were read in a Klett-Summerson colorimeter with a No. 54 filter. Each value obtained was corrected for the slight coloration which developed in a blank that contained no added lactate.

263 mμ, the absorption maximum of acrylyl pantetheine. In 2.3 hours the acrylyl pantetheine was essentially gone. The reaction mixture was deproteinized by addition of 0.2 ml. of 10 per cent perchloric acid. The precipitate was removed by centrifugation, and the supernatant solution was adjusted to pH 6.0 with 2 N potassium hydroxide. Purification of the product was carried out as with the synthetic lactyl pantetheine. Acrylyl pantetheine and β-alanyl pantetheine were prepared and assayed as previously described (2, 6).

The hydroxamate of β-hydroxypropionic acid was made by reacting propiolactone (Eastman Organic Chemicals) with 2.0 M hydroxylamine (pH 9.0). The hydroxamate was extracted into ethanol and then evaporated to dryness. The pure compound was recrystallized from *n*-butanol by the addition of water. This product had a melting point of 103–104° and the following elementary analysis:

$C_3H_7O_3N$

Calculated: C 34.3, H 6.66, N 13.32
Found: C 33.88, H 6.63, N 13.34

The zinc salt of β-hydroxypropionic acid was also made from propiolactone. Solid zinc oxide was added (in small aliquots with continuous stirring) to an aqueous solution of propiolactone, containing a few drops of phenol red indicator, until the indicator remained red. The solution was then evaporated to dryness. The zinc salt was extracted into ethanol and crystallized by the addition of water. In order to obtain the potassium salt, a solution of the zinc salt was passed over a small Dowex 50 column in the hydrogen form and then titrated with potassium hydroxide.

Pseudomonas sp. was grown aerobically in the following medium: potassium hydrogen phosphate, 1.5 gm.; sodium monobasic phosphate, 0.5 gm.; ammonium nitrate, 1.0 gm.; sodium propionate, 2.0 gm.; yeast extract, 0.5 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; and distilled water to a total volume of 1 liter. Cultures were harvested after 24 to 48 hours at 32°. The bacteria were separated by centrifugation and washed with distilled water. They were stored at −20°.

Cell-free extracts of this organism were prepared by suspending 20 gm. of cells (wet weight) in 45 ml. of 0.01 M potassium phosphate buffer, pH 7.5, and disrupting them by sonic oscillation for 2 periods of 5 minutes each in a Raytheon 10 kc. oscillator Magnetostrictor. The particulate material was removed by centrifugation for 15 minutes at 18,000 × *g* in a Servall angle centrifuge. The supernatant solution, containing 45.6 mg. of protein per ml., was stored at −20°.

Ammonium sulfate fractionation of the extract was done at 0°. To 40.0 ml. of extract were added 3.0 ml. of 1.0 M triethanolamine hydrochloride buffer, pH 7.5; 20.4 gm. of solid ammonium sulfate were added with constant stirring, bringing the concentration to 0.75 saturation. After 15 minutes equilibration, the precipitate was removed by centrifugation. To the supernatant solution was added solid ammonium sulfate to bring the final concentration to 0.95 saturation. After equilibration the precipitate was removed by centrifugation and dissolved in 9.0 ml. of 0.05 M triethanolamine hydrochloride buffer, pH 7.5. This solution was dialyzed at 0° against 2.0 liters of 0.01 M potassium phosphate buffer, pH 7.5, for 24 hours. The solution, containing 2.8 mg. of protein per ml., was stored at −20°. It had lost 47 per cent of its activity in 3 months and all of it in 1 year. Fractionation of the crude extract after storage for 1 year showed that it, too, had become inactive.

Pigeon heart muscle extract was prepared from an acetone powder as previously described (6). To 11.5 ml. of extract, containing 46.3 mg. of protein per ml., was added 0.9 ml. of 1.0 M triethanolamine hydrochloride buffer, pH 7.5. With constant stirring at 0°, 7.5 gm. of solid ammonium sulfate were added to bring the saturation to 0.60. The precipitate was removed by centrifugation. To the supernatant solution was added sufficient ammonium sulfate to bring the solution to 0.8 saturation. The precipitate was separated by centrifugation and dissolved in 10.0 ml. of 0.05 M triethanolamine hydrochloride buffer, pH 7.5. The solution was dialyzed overnight against 3 liters of 0.01 M potassium phosphate, pH 7.5, at 4°. The solution, containing 13.0 mg. of protein per ml., was stored at −20°.

The diaphorase preparation was prepared from sonic extracts of *Clostridium propionicum* grown on α-alanine. Conditions for growing this organism and preparing cell-free extracts have been presented (6). Acetic acid, 1.0 M, was added dropwise with constant stirring at 0° to 20.0 ml. of extract (containing 40 mg. of protein per ml.), bringing the pH to 4.8. The precipitate was

separated by centrifugation at 18,000 × *g*. It was immediately dissolved in 10 ml. of 0.1 M triethanolamine hydrochloride buffer, pH 7.0. The solution was dialyzed overnight against 2 liters of 0.01 M potassium phosphate buffer, pH 7.0. The solution, containing 29 mg. of protein per ml., was stored at −20°.

Conditions for growing and preparing cell-free extracts of *Clostridium kluyveri* have been presented (15).

Protein determinations were done by the method of Gornall *et al.* (16). The method of Fridovich and Handler (17) was utilized for assay of mercaptans with the modification that one-fourth the suggested concentration of dithizone was used.

Lactic dehydrogenase was purchased from Worthington Biochemical Corporation and assayed by the method of Kornberg (18). 2,3,5-Triphenyltetrazolium chloride was purchased from Schwarz Laboratories, Inc. Pantetheine, CoA, DPN, and DPNH were commercial preparations.

RESULTS

Spectrophotometric Evidence of Conversion of α,β-Unsaturated Thiolester to Saturated Thiolester by Pseudomonas sp.—α,β-Unsaturated thiolesters of pantetheine have an ultraviolet absorption maximum at 263 mμ, whereas the saturated thiolesters have a peak at 232 to 235 mμ (19). As described previously (6), the conversion of an unsaturated to a saturated thiolester of pantetheine can be followed by measuring the decrease in absorbance at 263 mμ. When acrylyl pantetheine was incubated at room temperature with an ammonium sulfate fraction of *Pseudomonas* sp. (Fig. 2), there was a rapid decrease of absorbance at 263 mμ, indicating the disappearance of acrylyl pantetheine. Addition of ammonium chloride did not increase the rate of disappearance. There was no reaction with a boiled enzyme preparation. Analysis at the end of the reaction showed an ultraviolet absorption spectrum with a single peak at 232 mμ that was indistinguishable from the spectrum of synthetic lactylpantetheine. Two facts suggested that this reaction differed from the acrylyl-CoA aminase reaction (6). The rate of the reaction is independent of ammonium salt concentration, and the kinetics of the reaction are zero order over the concentration range that was studied.

Demonstration of Hydroxamic Acid Derivative of Lactyl pantetheine—Since it was shown that acrylyl pantetheine is probably converted to a saturated thiolester, the addition of water across the double bond of the thiolester was postulated. Such an addition could lead to the formation of either β-hydroxypropionyl pantetheine or lactyl pantetheine (α-hydroxypropionyl pantetheine). To test these two possibilities the following experiment was done. To each of four tubes were added 50 μmoles of triethanolamine hydrochloride buffer (pH 7.5), 2.0 μmoles of acrylyl pantetheine, and water to a total volume of 0.75 ml. In addition, tubes 2 and 3 contained 1.4 mg. of protein of the *Pseudomonas* sp. preparation, and tubes 3 and 4 contained 100 μmoles of ammonium chloride. The samples were incubated at 29°. Aliquots from tubes 2 and 3 were checked spectrophotometrically to observe the decrease in absorbance at 263 mμ. The reaction proceeded to the complete disappearance of acrylyl pantetheine (as judged by the decrease in absorbance). At 75 minutes, 1.0 mmole of neutral hydroxylamine was added to each of the tubes and the resultant hydroxamates were chromatographed. To serve as markers, 1.0 μmole of the hydroxamic acid derivatives of lactate, β-hydroxypropionate, β-alanine, and a mixture of the three were included. The solvent system containing second-

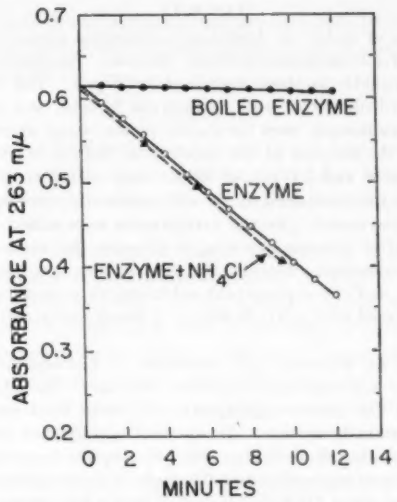

FIG. 2. Decomposition of acrylyl pantetheine by an ammonium sulfate fraction of *Pseudomonas* sp. 1.5-ml. cuvettes with 1.0-cm. light path were used. Each cuvette contained 50 μmoles of triethanolamine hydrochloride buffer, pH 7.5; 0.1 μmole of acrylyl pantetheine; where indicated, 1.4 mg. of enzyme protein, boiled enzyme, and 100 μmoles of ammonium chloride; and water in a total volume of 1.0 ml. A blank contained no acrylyl pantetheine. Absorbance was followed at 263 mμ.

ary butanol and formic acid was employed. Development of the chromatogram showed that sample 1 contained one hydroxamate derivative with the R_F of acrylate; samples 2 and 3 each contained a single hydroxamate with the R_F of lactate; sample 4 contained a dark spot with the R_F of acrylate as well as a faint spot with the R_F of β-alanine. Nonenzymatic conversion of acrylyl pantetheine to β-alanyl pantetheine in the presence of ammonia is known to occur (6). The mixture of the three hydroxamates was resolved very well by this system, lactate having an R_F of 0.55 as compared to 0.43 and 0.13 for β-hydroxypropionate and β-alanine respectively. These results suggested that lactyl pantetheine was the only product of the enzymatic hydration of acrylyl pantetheine.

Evidence for Production of Lactate by Hydrolysis of New Thiolester—Four μmoles of the purified, enzymatically produced thiolester were subjected to alkaline hydrolysis. This solution was neutralized and used in the experiments described below.

The hydrolyzed thiolester solution was analyzed for the presence of lactic acid, with the use of the lactate assay with 2,3,5-triphenyltetrazolium chloride. In both Experiments No. 1 and 2 (Table II, column *A*) there was the same amount of 2,3,5-triphenyltetrazolium chloride reduced in the complete system with the hydrolyzed product as with an equivalent quantity of a potassium lactate control. Omission of lactic dehydrogenase completely blocked the 2,3,5-triphenyltetrazolium chloride reduction in both cases. The enzymatic product, which had not been hydrolyzed, and potassium β-hydroxypropionate led to no color formation. Because the product of this reaction was to be used in further studies, the insoluble formazan was removed from the solution by centrifugation.

In order to establish that the oxidation product of the above reactions was pyruvate, the hydrazone derivative was made. One-fifth of a milliliter of 0.5 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid was added to aliquots of the supernatant

TABLE II

A. Assay of lactate in hydrolyzed enzymatic product by reduction of 2,3,5-triphenyltetrazolium chloride. Incubation conditions were exactly as those described for Fig. 1. The enzymatic product (hydrolyzed or not), potassium lactate, and potassium β-hydroxypropionate were incubated in the assay system where indicated. At the end of the incubation, 0.2 ml. of 10 per cent perchloric acid and 1.0 ml. of water were added to each. The precipitates were removed by centrifugation and the supernatant solutions were saved. To the precipitates were added 2.7 ml. of ethanol. After thorough mixing to dissolve the reduced 2,3,5-triphenyltetrazolium chloride, the protein was removed by centrifugation, and the supernatant solutions were read in a colorimeter equipped with a No. 54 filter. A blank contained no added lactate.

B. Assay for pyruvate after oxidation of hydrolyzed enzymatic product by 2,3,5-triphenyltetrazolium chloride. The supernatant solutions of the above experiments (*A*) were neutralized with 1.0 M potassium hydroxide. Each cuvette contained 200 μmoles of potassium phosphate buffer, pH 7.5; aliquots from the supernatant solutions equivalent to 0.05 μmole of the original substrate added; 0.2 μmole of DPNH; 1.0 unit of lactic dehydrogenase; and water to a volume of 1.0 ml. Blanks contained aliquots of the supernatant solutions from the blanks of the experiments in (*A*). The decrease in absorbance at 340 mμ caused by the addition of lactic dehydrogenase was measured. The values for pyruvate were corrected to the original volumes of the supernatant solutions.

Experiment No.	Sample	Amount tested	*A* Lactate by reduction of 2,3,5-triphenyltetrazolium chloride	*B* Pyruvate by DPNH oxidation
		μmoles	*μmoles*	*μmoles*
1	Enzymatic product (hydrolyzed)			
	Complete system	0.1	0.11	0.1
	Minus lactic dehydrogenase	0.1	0	0
	Enzymatic product (not hydrolyzed)	0.1	0	0
	Potassium lactate			
	Complete system	0.1	0.1	0.1
	Minus lactic dehydrogenase	0.1	0	0
	Potassium β-hydroxypropionate	0.1	0	0
2	Enzymatic product (hydrolyzed)	0.5	0.5	0.55
	Potassium lactate	0.5	0.54	0.55

solutions of Experiment No. 2, Table II. The samples were neutralized by addition of 1.0 N potassium hydroxide and extracted twice with ethyl acetate. The aqueous layer was acidified to pH 2 with 2 N hydrochloric acid, and the extraction was repeated. The second ethyl acetate extract, containing the acid hydrazones, was chromatographed on paper impregnated with 0.1 M potassium phosphate buffer (pH 7.5) in a solvent system consisting of water-ethanol, 17:83 (20). The oxidation products of both the hydrolyzed enzymatic product and potassium lactate gave a hydrazone spot at R_F 0.37.

In order to establish the stoichiometry of lactate oxidation in the 2,3,5-triphenyltetrazolium chloride experiments, aliquots of the supernatant solutions from those experiments were assayed for pyruvate by measuring the oxidation of DPNH in the presence of added lactic dehydrogenase. As shown in Table II, column *B*, there was good agreement between the amount of pyruvate present in the supernatant solutions and the amount of lactate that had been present (as measured by 2,3,5-triphenyltetrazolium chloride reduction). This was true in the case of the experiments with the hydrolyzed enzymatic product as well as with potassium lactate.

Another method by which the oxidation of lactate by lactic dehydrogenase can be brought to completion is that described by Horn and Bruns (21); 0.05 and 0.1 μmole of the hydrolyzed enzymatic product led to 0.06 and 0.11 μmole of DPNH formation, using their procedure for the quantitative assay of lactate. β-Hydroxypropionate led to no DPNH formation.

Irreversibility of Lactyl-CoA Hydrase Reaction—1. Spectrophotometric studies. With experimental conditions similar to those in Fig. 2, 2.0 μmoles of synthetic lactyl pantetheine were incubated with enzyme. There was no increase in absorbance at 263 mμ to indicate formation of acrylyl pantetheine by a reversal of the lactyl-CoA hydrase reaction.

2. Coupling acrylyl-CoA hydrase reaction to acrylyl-CoA aminase reaction. The equilibrium of the aminase reaction greatly favors the formation of β-alanyl pantetheine (6). If the acrylyl-CoA hydrase reaction were reversible, the possibility existed that the two reactions might be coupled. In the presence of lactyl thiolester and excess ammonium chloride this would result in the accumulation of β-alanyl thiolester. Incubation experiments with both synthetic lactyl pantetheine and lactyl-CoA were done. Chromatographic analysis of the hydroxamate derivatives of the reaction products showed no β-alanylhydroxamate. Hydrolysis of the reaction products and analysis for free β-alanine (a more sensitive method) by chromatography and spraying with ninhydrin also gave negative results.

Lactyl-CoA Hydrase in Pigeon Heart Muscle—Of the several sources of enzyme examined for the ability to metabolize acrylyl pantetheine, pigeon heart muscle was the only one, other than *Pseudomonas* sp., that could catalyze the formation of lactyl pantetheine. Experiments identical with those already described, but done with 6.5 mg. of protein from the ammonium sulfate fraction of pigeon heart, demonstrated (*a*) an enzymatic decomposition of acrylyl pantetheine independent of ammonium

TABLE III

Lactyl-CoA deacylase in extracts of C. propionicum and C. kluyveri

Each tube contained 50 μmoles of potassium phosphate buffer, pH 7.0; 0.5 μmole of lactyl-CoA; 4.0 mg. of protein (either the crude extract of *C. propionicum* or of *C. kluyveri*); and water to 0.5 ml. The blanks contained no lactyl-CoA. Incubations were done at 30°. Aliquots, equivalent to 0.05 μmole of the original thiolester, were removed and assayed for mercaptan (17) and for free lactate (21) at the designated times. Values for the blanks were subtracted.

Time	*C. propionicum*		*C. kluyveri*	
	Mercaptan	Lactate	Mercaptan	Lactate
minutes	*μmoles*	*μmoles*	*μmoles*	*μmoles*
0	0.003	0	0.003	0
30	0.03		0.041	
60	0.04		0.052	0.056
120	0.05	0.058		

salts, (*b*) a reaction product which had an ultraviolet spectrum identical to lactyl pantetheine, and (*c*) a hydroxamic acid derivative of the product of the reaction which was indistinguishable from the hydroxamate of lactyl pantetheine in two solvent systems (A and B, Table I). Extracts of the following microorganisms failed to show acrylyl-CoA hydrase activity under our experimental conditions: *Clostridium propionicum*, *Clostridium kluyveri*, *Clostridium sticklandii*, *Micrococcus lactilyticus*, *Propionibacterium pentosaceum*,[3] and 2 strains of aerobes isolated with β-alanine as the sole carbon source in enrichment cultures.

Lactyl-CoA Deacylase—After it was shown that the product of the enzymatic hydration of acrylyl pantetheine is lactyl pantetheine, an attempt was made to study the further metabolism of the thiolester of lactic acid. For this study lactyl-CoA was chosen because it is probably the natural substrate.

The 2,3,5-triphenyltetrazolium chloride reduction assay for lactate (Fig. 1) was adapted as an assay for DPN-linked lactyl-CoA dehydrogenase activity. For this purpose lactic dehydrogenase was omitted and lactyl-CoA was substituted for free lactate; 0.1 μmole of lactyl-CoA was used in the assay. With this procedure, an extract containing lactyl-CoA dehydrogenase activity would lead to the reduction of 2,3,5-triphenyltetrazolium chloride in the absence of lactic dehydrogenase. By including a parallel experiment with lactic dehydrogenase present deacylase activity would be demonstrated by increased dye reduction due to the oxidation of free lactate. Appropriate blanks containing free lactate and no substrate were also included.

There was no evidence of lactyl-CoA dehydrogenase or deacylase activity in crude extracts of *Pseudomonas* sp. or pigeon heart muscle using this procedure. Extracts of *C. propionicum* and *C. kluyveri*, however, did catalyze the reduction of 2,3,5-triphenyltetrazolium chloride in the presence of lactic dehydrogenase. There was no reduction of this substance in the absence of lactic dehydrogenase. This suggestive evidence for deacylase activity was confirmed by the experiments shown in Table III. Extracts of both organisms catalyzed the liberation of equivalent amounts of mercaptan and lactate from lactyl-CoA. Similar experiments with boiled extract preparations of each showed no deacylase activity.

DISCUSSION

Lactate has been implicated a number of times in propionate metabolism (22–29), and an activated form has been postulated (27). Until now, however, there has been no direct evidence for such an activated compound. Although the enzyme which catalyzes the formation of lactyl pantetheine is present in a pseudomonad and pigeon heart muscle, its general significance to propionate metabolism is still not clear. Study of the further metabolism of lactyl-CoA was undertaken with the hope of demonstrating pyruvyl-CoA as the next metabolite. Lactyl-CoA dehydrogenase activity was not discovered in our experiments, but extracts of two unrelated organisms were found to contain a deacylase of lactyl-CoA. Although deacylation is one pathway for the metabolism of lactyl-CoA, it cannot be assumed to be the only one until a more extensive survey is made.

SUMMARY

Acrylyl coenzyme A hydrase, an enzyme which catalyzes the hydration of acrylyl pantetheine to form lactyl pantetheine, was demonstrated in extracts of *Pseudomonas* sp. and pigeon heart muscle. The enzymatically formed lactyl pantetheine was identified by spectroscopic and chromatographic comparison with synthetic lactyl pantetheine; lactate was identified as the product of alkaline hydrolysis by showing its conversion to pyruvate in the presence of lactic dehydrogenase and diphosphopyridine nucleotide. The enzymatic reaction was found to be irreversible under our experimental conditions.

Lactyl coenzyme A dehydrogenase activity was not found in extracts of *Pseudomonas* sp., pigeon heart muscle, *Clostridium propionicum*, or *Clostridium kluyveri*. However, an enzyme which catalyzes the deacylation of lactyl coenzyme A was demonstrated in extracts of the last two organisms.

REFERENCES

1. Stadtman, E. R., *Bull. Soc. Chim. biol.*, **37**, 931 (1955).
2. Stadtman, E. R., *J. Am. Chem. Soc.*, **77**, 5765 (1955).
3. Stadtman, E. R., *Federation Proc.*, **15**, 360 (1956).
4. Stadtman, E. R., and Vagelos, P. R., *International symposium on enzyme chemistry, Tokyo and Kyoto*, 1957, Pan-Pacific Press, Tokyo, 1958, p. 86.
5. Vagelos, P. R., and Stadtman, E. R., *Abstracts of papers of the 131st meeting of the American Chemical Society*, Washington, 1957, p. 24C.
6. Vagelos, P. R., Earl, J. M., and Stadtman, E. R., *J. Biol. Chem.*, **234**, 490 (1959).
7. Rendina, G., and Coon, M. J., *J. Biol. Chem.*, **225**, 523 (1957).
8. Giovanelli, J., and Stumpf, P. K., *J. Biol. Chem.*, **231**, 411 (1958).
9. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184**, 769 (1950).
10. Stadtman, E. R., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. III*, Academic Press, Inc., New York, 1957, p. 934.
11. Wieland, T., and Köppe, H., *Ann. Chem. Liebigs*, **581**, 1 (1953).
12. Neilands, J. B., *J. Biol. Chem.*, **199**, 373 (1952).
13. Quastel, J. H., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. IV*, Academic Press, Inc., New York, 1957, p. 334.
14. Wieland, T., and Köppe, H., *Ann. Chem. Liebigs*, **588**, 15 (1954).
15. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1085 (1949).
16. Gornall, A. G., Burdawill, C. J., and David, M. M., *J. Biol. Chem.*, **177**, 751 (1949).
17. Fridovich, I., and Handler, P., *Anal. Chem.*, **29**, 1219 (1957).
18. Kornberg, A., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. I*, Academic Press, Inc., New York, 1957, p. 441.
19. Lynen, F., *Federation Proc.*, **12**, 683 (1953).
20. Kun, E., and Hernandez, M. G., *J. Biol. Chem.*, **218**, 201 (1956).
21. Horn, H. D., and Bruns, F. H., *Biochim. et Biophys. Acta*, **21**, 378 (1956).
22. Cardon, B. P., and Barker, H. A., *Arch. Biochem.*, **12**, 165 (1947).
23. Johns, A. T., *J. Gen. Microbiol.*, **5**, 326 (1951).
24. Johns, A. T., *J. Gen. Microbiol.*, **6**, 123 (1952).
25. Mahler, H. R., and Huennekens, F. M., *Biochim. et Biophys. Acta*, **11**, 575 (1953).
26. Leaver, F. W., Wood, H. G., and Stjernholm, R., *J. Bacteriol.*, **70**, 521 (1955).
27. Elsden, S. R., Volcani, B. E., Gilchrist, F. M. C., and Lewis, D., *J. Bacteriol.*, **72**, 681 (1956).
28. Ladd, J. N., and Walker, D. J., *Biochem. J.*, **69**, 29P (1958).
29. Walker, D. J., and Ladd, J. N., *Biochem. J.*, **69**, 29P (1958).

[3] Dried cell preparations of *Micrococcus lactilyticus* and *Propionibacterium pentosaceum* were most kindly provided by Dr. S. F. Carson.

The Biosynthesis of Lycopene in Tomato Homogenates*

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It has been demonstrated that radioactive lycopene results from the addition of labeled acetate to intact, green, ripening tomatoes (1, 2). In order to study more closely the metabolic pathway involved in the formation of this carotenoid, attempts to prepare a cell-free system which could synthesize lycopene were carried out. Labeled mevalonic acid could serve as a source of lycopene carbon in tomato homogenates, but acetate and a number of other low molecular weight substances gave negative results. The preparation of tomato homogenates, the cofactor requirements, and other conditions for lycopene biosynthesis are described in this paper.

EXPERIMENTAL

Materials—Adenosine triphosphate, and di- and triphosphopyridine nucleotide were obtained from the Pabst Laboratories, glutathione from the Schwarz Laboratories, and tetrasodium ethylenediaminetetracetate (Versene) from the Bersworth Chemical Company.

Two preparations of 2-C^{14}-mevalonic acid, as the N,N′-dibenzylethylenediamine salt, were obtained from the Isotopes Specialties Company. The first, which was used for many of the experiments reported here, had a melting point of 123–124° and contained approximately 10 per cent of a radioactive contaminant. The second sample had a melting point of 125–126° and was radiochemically pure as determined in two different solvent systems (3). The second of the two samples gave a greater incorporation of radioactivity into lycopene. Free mevalonic acid was released from its salt according to Hoffman *et al.* (4). We are indebted to Dr. James Sprague of Merck Sharp and Dohme for a generous sample of unlabeled mevalonic acid and a sample of mevalonic acid of low specific activity. Other radioactive substances were obtained from various commercial sources.

Commercial grade, selected, fresh tomatoes were obtained from the Imperial Valley, California. Preliminary experiments suggested that tomatoes which had just reached full ripeness gave optimal results, and these were therefore used in these studies.

The muscle extract used as a source of glycolytic enzyme was a 30 to 70 per cent ammonium sulfate fraction. When assayed for pyruvate with phosphoglycerate and ADP (5), 1 ml. was found to produce 10 μmoles of pyruvate in 1 hour.

Methods—Since the pH of whole ground tomato is 3 to 4, a number of procedures were tested for rapid neutralization during preparation of tomato homogenates. The method finally developed which gave the best and most reproducible results is given here. Tomatoes were cooled to 5° and rinsed in mild soapy water, distilled water, 70 per cent ethanol, and distilled water. The skin was rapidly removed with a single edge razor as close to the outer layer as possible, and the tomato was cut into quarters and placed in cold homogenizing medium containing 0.2 M Tris[1] buffer, pH 8.2, 0.02 M nicotinamide, and 0.002 M Versene. The seed pocket content was removed by hand, and the hard core pieces at the ends of each quarter were cut off and discarded. The remaining parenchyma of the tomato was rinsed briefly in cold homogenizing medium and 30 gm. of tomato tissue were ground for 30 seconds in a Potter-Elvehjem, Teflon pestle homogenizer with 10 ml. of medium. The pestle-tube clearance was 1.5 mm. This procedure yielded a viscous homogenate with a pH of 7.8. No whole cells could be seen under the microscope.

After incubation, 5 ml. of carrier in the form of commercial tomato paste containing 2 to 3 mg. of lycopene were added to the flask with the aid of 20 ml. of anhydrous methanol. The tomato paste was dehydrated before use by two treatments with equal volumes of methanol. The incubation mixture and the tomato paste were thoroughly mixed and centrifuged, and the supernatant solution was discarded. The pellet was washed again with 30 ml. of anhydrous methanol. Lycopene and other lipides were then extracted from the particulate material by three treatments with 30 ml. of carbon tetrachloride-methanol (1:1), thorough mixing, and centrifugation. The carbon tetrachloride-methanol extracts were washed 12 times with 150 ml. of water by brief stirring and decantation to remove methanol and water-soluble radioactive substrates. The carbon tetrachloride solution was dried with anhydrous sodium sulfate and filtered, and the solvent was removed by heating briefly on a steam bath under a stream of nitrogen.

Pure lycopene was obtained by absorption chromatography on a 3.5 × 8-cm. column of magnesium oxide-Super-Cel, 1:1. The lipide residue in 5 ml. of benzene was added to the column, and 100 ml. of petroleum ether (30–60°) followed by 100 ml. of 30 per cent acetone in petroleum ether were passed through the column under nitrogen pressure. The column was extruded, and the lycopene band was removed and extracted five times with 20 ml. of a petroleum ether (30–60°)-benzene-methanol (5:4:1) solution. The extract was filtered and dried in a vacuum under

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[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

nitrogen, and lycopene was crystallized three times from hot benzene-methanol. The crystals were washed four or five times with 2 ml. of hot methanol and dried. The purity and the percentage of recovery of lycopene were measured from time to time with the spectrophotometric method of Zscheile and Porter (6). The recovery of lycopene including carrier was 80 to 90 per cent, and the material was 100 per cent pure as judged by spectrophotometric assay. Melting points, which were occasionally taken, compared very favorably with published values. In at least half of the experiments in which radioactive lycopene was formed, the crystalline material was saponified, then chromatographed, crystallized, and washed again without change in specific activity.

Partition of the lipides in the carbon tetrachloride extract was carried out according to Kuhn and Brockmann (7). Lycopene and other lipide samples were plated and counted directly under conditions of negligible self-absorption.

RESULTS

C^{14}-Acetate, Glycolytic and Tricarboxylic Acid Cycle Intermediates as Substrates—A large number of experiments testing acetate and other radioactive substrates with tomato homogenates for lycopene biosynthesis under a variety of conditions was carried out. A few of these are shown in Table I. In no case was radioactive lycopene formed, although considerable radioactivity was incorporated into the total lipide fraction extracted by carbon tetrachloride, particularly when 2-C^{14}-acetate was used as tracer.

The distribution of radioactivity in this lipide fraction from 2-C^{14}-acetate was determined with the results shown in Diagram 1. Very little activity was present in the neutral lipides, but a considerable amount of activity was found in the ether-soluble acid fraction. Most of the activity, however, was water-soluble after saponification and was probably due to esterified acetate.

2-C^{14}-Mevalonic Acid as Substrate—When 2-C^{14}-mevalonic acid was tested as a precursor for lycopene biosynthesis in tomato homogenates, radioactivity was incorporated into the isolated, purified carotenoid (Table II). Omission of ATP resulted in a lowering of activity to about one-quarter, and omission of DPN and TPN, or glutathione, to one-third to one-half of that in the complete system. Replacement of air by nitrogen caused a great decrease in lycopene formation from mevalonic acid. The most favorable pH in Tris buffer was found to be about 8.0 (Fig. 1). Above this value, results were inconclusive and not reproducible. A few preliminary experiments were carried out with phosphate buffer which gave lower values. The best in-

TABLE I

Incorporation of radioactivity into lipides and lycopene from acetate, glycolytic, and tricarboxylic acid substrates

Each flask contained 20 μmoles of radioactive substrate (10 μmoles each in Experiment 6), specific activity 310,000 c.p.m. per μmole; 1 μmole of TPN, 100 μmoles of GSH; 10 μmoles of ATP; 50 μmoles of $MgSO_4$; 9 ml. of a homogenate prepared with an equal volume of tomato tissue and 0.2 M phosphate buffer, pH 8.2, in a final volume of 10 ml. The pH of the mixture was 7.0. Incubation was in air at 25° for 12 hours.

Experiment No.	Substrate	Lipides	Lycopene
		c.p.m.	*c.p.m.*
1	2-C^{14}-acetate	188,000	0
	2-C^{14}-acetate, boiled homogenate	0	0
2	3-C^{14}-pyruvate	26,000	0
3	1,5-C^{14}-citrate	9,000	0
4	2,3-C^{14}-fumarate	60,000	0
5	Uniformly labeled C^{14}-glucose	66,000	0
6	2-C^{14}-acetate and 3-C^{14}-pyruvate	260,000	0

DIAGRAM 1

Distribution of activity in lipide fractions from 2-C^{14} acetate

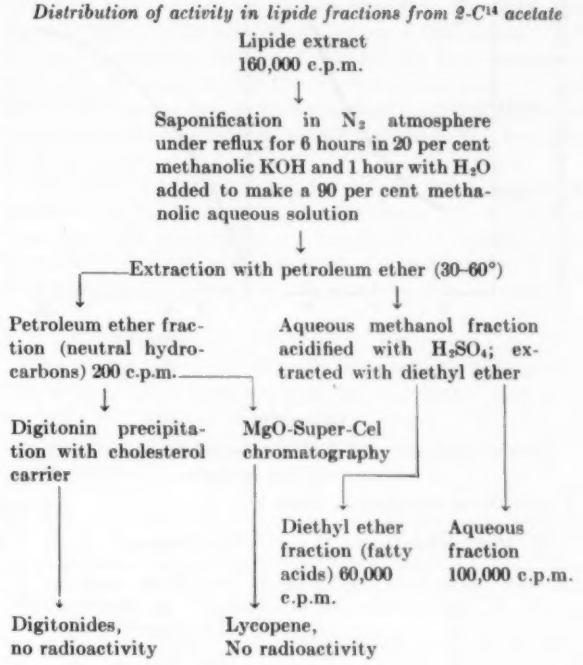

TABLE II

Incorporation of radioactivity into lipides and lycopene from 2-C^{14}-mevalonic acid

The complete system contained 5 μmoles of sodium 2-C^{14}-mevalonate, 250,000 c.p.m. per μmole; 20 μmoles of ATP; 1 μmole of DPN; 1 μmole of TPN; 20 μmoles of GSH; 50 μmoles of $MnSO_4$; and 9 ml. of homogenate prepared with Tris buffer as described in "Methods" in a final volume of 10 ml. Incubation was in air at 25° for 12 hours.

Additions	Lipides	Lycopene
	c.p.m.	*c.p.m.*
Experiment 1		
Complete system	50,000	270
Boiled homogenate	0	0
Zero time control	80	0
No ATP	6,000	90
No DPN, no TPN	30,000	140
No GSH	22,000	100
No Mn^{++}	94,000	220
N_2 instead of air	39,000	60
Experiment 2		
Complete system	40,000	350
Experiment 3		
Complete system	56,000	760

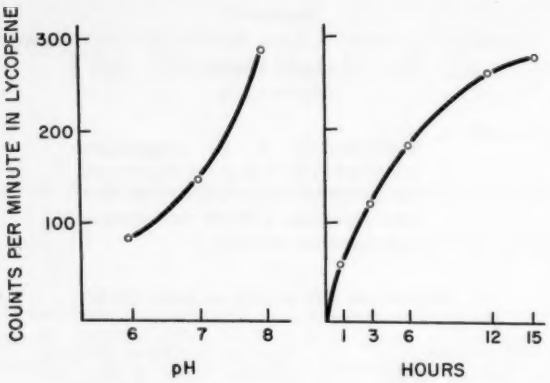

FIG. 1 FIG. 2

TABLE III

Effect of mevalonic acid concentration on incorporation of radioactivity into lycopene

Conditions as described in Table II.

Mevalonic Acid	Lycopene
μmoles	*c.p.m.*
1	65
5	270
10	310

TABLE IV

Effect of air and nitrogen on incorporation of radioactivity into lipides and lycopene

The complete system was as described in Table II.

Atmosphere	Additions	Lipides	Lycopene
		c.p.m.	*c.p.m.*
Experiment 1			
Air	Complete	46,000	270
Air	Complete + 1 ml. muscle extract + 50 μmoles 3-phosphoglycerate	75,000	165
Nitrogen	Complete	39,000	60
Nitrogen	Complete + 1 ml. muscle extract + 50 μmoles 3-phosphoglycerate	51,000	50
Experiment 2			
Air	Complete	45,000	270
Air	Complete + 10 μmoles methylene blue	3,500	0
Nitrogen	Complete	31,000	65
Nitrogen	Complete + 10 μmoles methylene blue	4,400	0
Nitrogen	Complete + 10 μmoles indophenol	3,900	0

cubation time was 12 to 15 hours (Fig. 2). The effect of varying the concentration of mevalonic acid is shown in Table III.

Since the replacement of air by nitrogen greatly reduced the incorporation of radioactivity into lycopene, attempts were made to determine whether oxygen was necessary for regeneration of ATP or for terminal electron transport. Addition of an ATP-generating system consisting of a muscle extract and phosphoglycerate did not replace the requirement for air and caused a decrease in activity when air was present (Table IV, Experiment 1). When methylene blue or indophenol were added (Experiment 2) complete inhibition of lycopene biosynthesis occurred both in air and nitrogen. Thus the need for oxygen remains unexplained.

TABLE V

Distribution of activity in lipide fractions from 2-C^{14}-mevalonic acid

Fraction	Activity
	c.p.m.
Lipide extract	48,000
Petroleum ether extract	16,000
Digitonides	300
Lycopene	320
Fatty acids	4,000
H_2O-soluble after saponification	8,000

TABLE VI

Activity in lipides and lycopene after centrifugation of homogenate

Centrifugation was at 5000 × *g* for 5 minutes. Additions and incubations were as described in Table II.

Fraction	Lipides	Lycopene
	c.p.m.	*c.p.m.*
Whole homogenate	27,000	295
Whole homogenate, allowed to stand 15 minutes at 5° before incubation	23,000	190
Pellet, washed once with 0.02 M Tris buffer, pH 7.8	12,000	210
Supernatant	16,000	0

Partition of a lipide extract obtained after incubation of mevalonic acid with a tomato homogenate is shown in Table V. In contrast to the results obtained with acetate, the lipides which were extracted with petroleum ether after saponification contained a considerable portion of radioactivity, and the sterols precipitated by digitonin had as much activity as lycopene.

Attempts were made to determine which cellular fraction was the site of lycopene biosynthesis. However, centrifugation at very low speeds resulted in no clear-cut separation of nuclei and cell debris from the tomato homogenate because of the agglutinating particulate present in the mixture. After centrifuging at 2000 × *g* for 15 minutes, for example, the mixture contained a pellet at the bottom of the tube, a relatively clear solution in the center, and a solid particulate layer at the top. At 5000 × *g*, all of the visible particulate material was sedimented, leaving a clear supernatant solution. Activity was present only in the pellet (Table VI). Acetone powder extracts have been inactive.

DISCUSSION

The data presented have shown that labeled mevalonic acid can serve as a source of carbon for lycopene formation in tomato homogenates. For optimal incorporation of tracer, ATP, pyridine nucleotides, glutathione, manganese ion, and incubation in air are necessary. Evidence which established the purity of lycopene and the absence of radioactive contaminants was obtained by chromatography and crystallization and repetition of these procedures after complete saponification.

Although the homogenate could not be centrifuged to remove

unbroken cells, it is unlikely that more than traces of whole cells were present in the incubated mixtures. Not only was there an absence of whole cells under the microscope, but the marked stimulation of ATP, which would not be expected to cross cell walls, also argues against the presence of appreciable amounts of whole cells.

The amount of radioactivity incorporated into lycopene from mevalonic acid is actually a substantial fraction of the maximum that might be expected. According to Goodwin and Jamikorn, (8) 1 gm. (wet weight) of whole tomato produces about 2.5 μg. of lycopene during the ripening period of 12 days. About four-fifths of this, or 2 μg. per gm. are formed during the last 3 days. In the experiments with a broken cell system reported here, 6 gm. (wet weight) of nearly ripe tomato were incubated for 12 hours. On the assumption that a 12-hour incubation would allow the formation of one-sixth of that which occurs in 3 days, the maximal amount of lycopene that could be produced is 2 μg, or 3.7 mμmoles. The specific activity of the substrate was 250,000 c.p.m. per μmole, and 3.7 mμmoles are therefore equal to 925 c.p.m. Since each of the eight isoprene residues in lycopene should be derived from 1 mevalonic acid molecule, the upper limit of radioactivity to be expected in the biologically formed lycopene in the incubation mixtures is 7400 counts. This, however, assumes no dilution of radioactivity from endogenous unlabeled precursors, so that the observed incorporation of about 200 to 700 counts into lycopene isolated from the incubation mixtures is an appreciable amount compared to the maximum to be expected.

Mevalonic acid is thus a precursor not only for cholesterol (9), squalene (10, 11), rubber (12), and β-carotene (13) but for lycopene also. The preparation of a homogenate in which lycopene formation will occur may prove to be a powerful tool in the further study of this pathway for the synthesis of carotenoids.

SUMMARY

The preparation of homogenates of tomatoes which can synthesize radioactive lycopene from 2-C^{14}-mevalonic acid is described. For optimal incorporation of tracer, ATP, pyridine nucleotides, glutathione, manganese ion, and incubation in air are necessary.

REFERENCES

1. Zabin, I., *J. Biol. Chem.*, **226**, 851 (1957).
2. Shneour, E. A., and Zabin, I., *J. Biol. Chem.*, **226**, 861 (1957).
3. Hong, L., and Wright, L. D., *Biochim. et Biophys. Acta*, **26**, 212 (1957).
4. Hoffman, C. H., Wagner, A. F., Wilson, A. N., Walton, E., Shunk, C. H., Wolf, D. E., Holly, F. W., and Folkers, K., *J. Am. Chem. Soc.*, **79**, 2316 (1957).
5. Friedemann, T. E., and Haugen, C. E., *J. Biol. Chem.*, **147**, 415 (1943).
6. Zscheile, F. P., and Porter, J. W., *Ind. Eng. Chem., Anal. Ed.*, **19**, 47 (1947).
7. Kuhn, R., and Brockmann, H., *Z. physiol. Chem.*, **206**, 41 (1932).
8. Goodwin, T. W., and Jamikorn, M., *Nature*, **170**, 104 (1952).
9. Tavormina, P. A., Gibbs, M. H., and Huff, J. W., *J. Am. Chem. Soc.*, **78**, 4498 (1956).
10. Dituri, F., Gurin, S., and Rabinowitz, J. L., *J. Am. Chem. Soc.*, **79**, 2650 (1957).
11. Amdur, B. H., Rilling, H., and Bloch, K., *J. Am. Chem. Soc.*, **79**, 2646 (1957).
12. Park, R. B., and Bonner, J., *J. Biol. Chem.*, **233**, 340 (1958).
13. Braithwaite, G. D., and Goodwin, T. W., *Biochem. J.*, **67**, 13p (1957).

Studies on the Metabolism of Adipose Tissue

I. THE EFFECT OF INSULIN ON GLUCOSE UTILIZATION AS MEASURED BY THE MANOMETRIC DETERMINATION OF CARBON DIOXIDE OUTPUT*

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The interesting observations of Winegrad and Renold[1] (1, 2) on the effect of insulin on the metabolism of radioactive glucose by the rat epididymal fat pad *in vitro* were the stimuli to the study to be described here. The results of these workers clearly showed that insulin stimulated the conversion of glucose to fatty acids in this tissue concomitantly with a marked and preferential acceleration of the conversion of carbon 1 of glucose to CO_2. As a first approximation the effects of insulin which they observed can be expressed as a stimulation of the rate at which the following net reactions proceeded:

$$\text{Glucose} \rightarrow \text{2-acetyl CoA} + 4\ \text{DPNH} + 2\ CO_2 + 2 \sim \text{P} \quad (1)$$

$$\text{Glucose} + 1 \sim \text{P} \rightarrow \text{Pentose-PO}_4 + 2\ \text{TPNH} + CO_2 \quad (2)$$

$$n(\text{Acetyl-CoA}) + 2n - 2(\text{TPNH} + \text{DPNH}) \rightarrow \text{Fatty acid} \quad (3)$$

Neither the exact balance between these equations nor the relative needs for reduced tri- and diphosphopyridine nucleotide in Equation 3 were clearly evident from their results. However, consideration of these equations did suggest that, due to the participation of the phosphogluconate pathway (Equation 2), an even more marked output of CO_2 might occur during fat synthesis from glucose than one had previously suspected. We therefore deemed it worthwhile to study the effect of insulin on the total gas exchange of the epididymal fat pad. This may be done very simply by means of the Warburg manometric apparatus. The adipose tissue is placed in a vessel with Krebs-Ringer bicarbonate, without any KOH in the center well for the absorption of CO_2, and gassed with a mixture of 5 per cent CO_2-95 per cent air. If the oxygen consumption of the tissue equals its carbon dioxide output (R. Q. = 1), then no pressure change will be observed. If the carbon dioxide output exceeds the oxygen consumption, then a positive pressure will develop and the exact volume of the excess CO_2 produced can be measured. Conversely, if the respiratory quotient is less than 1, a negative pressure will develop. As will be seen from the results, this procedure has proved eminently satisfactory for the study of the action of insulin on the metabolism of the epididymal fat pad. As is so often the case, we found after our initial experiments that our use of this procedure was not an original one. Balmain *et al.* (3) in 1950 applied this method to a study of fat synthesis by slices of lactating mammary gland.

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[1] The senior author of this paper is indebted to Dr. Renold for the opportunity of discussing with him the contents of these papers before they were submitted for publication.

EXPERIMENTAL

Male rats of several strains have been employed successfully for the work described here. The majority of the results have been obtained with rats purchased from the Holtzman Company and fed *ad libitum* on Purina laboratory chow. The rats were killed by decapitation by means of the miniature guillotine developed by the Harvard Apparatus Company. Portions of the epididymal fat pad were immediately removed with as little handling as possible in a manner similar to that described by Winegrad and Renold (1). They were weighed on a torsion balance and placed directly into a Warburg vessel containing the medium to be employed for the experiment. In small rats the distal portion of the fat pad from each side has been employed. In larger rats a distal and a proximal piece have been obtained from each fat pad so that four pieces of tissue from one rat could be compared. The basic medium employed has been that of Krebs and Henseleit (4) to which glucose, and sometimes gelatin, have been added, as will be indicated in the presentation of the results. The pH of the basic medium after equilibration with a gas mixture containing 5 per cent CO_2 was 7.35 as measured with the glass electrode at room temperature. The temperature of the medium has been that of the room (23–25°) during the loading of the flasks, though a minimum of delay has been practiced in the mounting of the flasks on their manometers and placing them in the constant temperature bath (37.2°). In our initial experiments we have used successfully the conventional type of Warburg vessel of approximately 15 ml. total capacity with a center well and one side arm with a vented stopper. Since the center well is not employed in our experiments, we have had vessels with a total capacity of about 10 ml. constructed without a center well and containing one side arm with a vented stopper. The amount of tissue used ranged from 200 to 400 mg. when the larger size vessel was employed and 100 to 300 mg. with the smaller size. The gas phase in all cases has been a mixture of 5 per cent CO_2-95 per cent air which was bubbled through water before entering the flask. Gassing was continued for a period of about 5 minutes with slow shaking, and readings usually began within 10 to 15 minutes after insertion of the flasks in the water bath.

The insulin used was kindly furnished by Eli Lilly and Company and was a crystalline zinc insulin preparation, Lot No.

466368, which assayed 25 units per mg. Stock solutions of insulin which contained 30 units per ml. were made up in water which contained 2 drops of 0.1 N HCl per 5 ml. Such solutions were kept in the cold room and used for periods up to a month. Working solutions were prepared by a 1 to 10 dilution of the stock solution with Krebs-Ringer bicarbonate medium.

Glass redistilled water has been employed throughout for all solutions.

RESULTS

The results of a typical experiment are shown in Fig. 1. Two pieces of tissue, one from the right and one from the left epididymal fat pad of the same rat, were employed. Each was placed in a flask which contained the same basic Ringer-bicarbonate medium, but one contained glucose to yield a final concentration of 4 mg. per ml. and the other contained no glucose. Insulin was placed in the side arm of both flasks so that after tipping, the final concentration of insulin in the flask would be 0.1 unit (10^5 micro units) per ml. During the first 60 minutes both flasks showed pressure changes which indicated that more gas was being consumed than was evolved; in other words, the respiratory quotient of the tissue during the initial period was less than 1. At the end of 60 minutes insulin was tipped into the main compartment of the flask from the side arm. In the vessel which contained no glucose the rate of net gas exchange remained unaltered. In the flask containing glucose a positive pressure response to the insulin is evident within the first 10 minutes after the addition of insulin. Gas, presumably carbon dioxide, is being evolved more rapidly than oxygen is being consumed and the respiratory quotient has changed from a slightly negative value to a marked positive one. Once it is under way the rate of gas evolution is remarkably linear with time, and under suitable conditions we have observed that the rate will remain constant for at least 3 hours after the addition of insulin. This stimulation of gas production by the addition of insulin is consistent with the expected increase in CO_2 production that may be predicted from Equations 1 and 2. Now the experimental conditions employed here are nearly identical with those used in the studies reported by Winegrad and Renold (1, 2). In view of this fact and the results reported by these workers it seems reasonable to conclude that fatty acid formation is occurring simultaneously with the increased gas formation observed here. The fact that the rate of gas exchange in the absence of glucose is unaffected by the addition of insulin indicates that the respiratory processes of the tissue are unaffected by insulin added in the absence of glucose. In experiments to be published elsewhere, it has been found that the oxygen uptake of this adipose tissue proceeds at a linear rate and is not markedly affected by the addition of insulin either in the presence or absence of glucose. Similar results have been reported previously by Haugaard and Marsh (5).

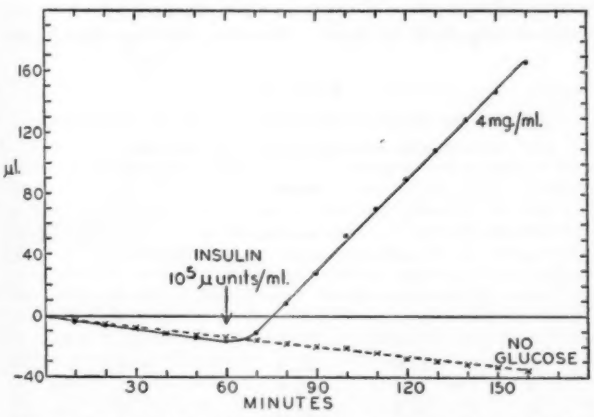

FIG. 1. The effect of insulin on the net gas exchange in the presence and absence of glucose. Flasks with a total volume of 13 to 15 ml. and a center well were employed. Each flask contained 2.9 ml. of Krebs-Ringer bicarbonate in the main chamber. At 60 minutes 0.1 ml. of an insulin solution in Krebs-Ringer bicarbonate which contained 3 units per ml. was tipped from the side arm to yield a final concentration in the vessel of 0.1 unit (10^5 micro units) per ml. In one flask glucose was added to yield a final concentration of 4 mg. per ml. The tissue in this flask weighed 218 mg. and was obtained from the left epididymal fat pad. The other flask contained no glucose and a piece of tissue which weighed 147 mg. from the right fat pad of the same rat. Temp. 37.2°.

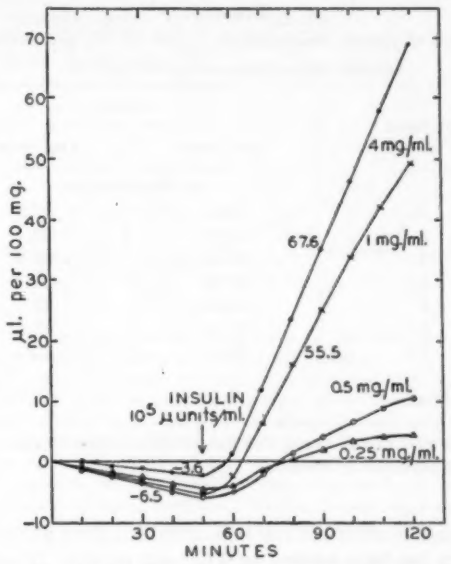

FIG. 2. The effect of variations in the glucose concentration on the response to insulin. Flasks with a total volume of 8 to 12 ml. without a center well were employed. The main compartment of each flask contained 1.35 ml. of Krebs-Ringer bicarbonate and glucose to give the concentration indicated in mg. per ml. The insulin solution, 0.05 ml., was tipped from the side arm at 50 minutes to give a final concentration of 10^5 micro units per ml. All tissue was obtained from a single rat. The weight and location of the tissue employed in each flask are as follows: 0.25 mg. per ml., 190 mg., right distal; 0.5 mg. per ml., 235 mg., right proximal; 1 mg. per ml., 193 mg., left distal; 4 mg. per ml., 251 mg., left proximal.

The effect of variations in the glucose concentration on the rate of the reaction stimulated by insulin is shown by the data presented in Fig. 2. Unlike the results presented in Fig. 1 where the directly observed readings were given, the data here are expressed in terms of ml. of gas exchange per 100 mg. of wet tissue. In this experiment four pieces of tissue from a single rat have been employed. The rate of the reaction is seen to increase as the glucose concentration in the medium increases. A positive response is obtained upon the addition of insulin as long as some glucose is in the medium. In other experiments of this

TABLE I

Effect of glucose concentration on rate of CO_2 production

Insulin concentration 0.1 unit per ml.

Experiment	Glucose	
	1 mg. per ml.	4 mg. per ml.
	*μl. CO_2/100 mg./hr.**	
1	30.0	27.3
2	34.0	60.1
3	21.7	22.9
4	17.6	26.2
5	19.8	22.7
6	11.8	13.3
7	38.2	50.3
Mean	24.7	31.8

* Expressed as net change in gas evolution that occurs before and after addition of insulin.

type an insulin effect has still been noted when the glucose concentration has been lowered to 0.125 mg. per ml. Invariably, however, in the complete absence of added glucose no response is obtained and the results are of the type shown in Fig. 1. It should be noted that at lower glucose concentrations not only is the initial rate of gas evolution slow, but the reaction fails to remain linear with time. This is particularly noticeable when the experiments are carried out in the smaller vessels where the ratio of total medium to tissue is reduced, as is the case for the experiment shown in Fig. 2. The greatest effect of changes in glucose concentration on the rate is in the region from 0 to 1 mg. per ml. The change in rate obtained by increasing the glucose concentration from 1 to 4 mg. per ml. is less striking, as is to be seen from Fig. 2 and the data presented in Table I. It is of interest to note that in the absence of insulin the tissue appears to be markedly unresponsive to the presence of glucose in the medium. Even in the presence of 4 mg. per ml. of glucose the tissue continues to maintain an R. Q. which is less than 1.

For the comparison of data obtained from experiments of this type we have found it convenient to express our results in terms of μl. of gas exchange per 100 mg. wet weight of tissue per hour. We have also chosen to express our results in terms of the net change in gas evolution as calculated from the differences in rates before and after the addition of insulin. That this procedure has definite advantages will be seen when we deal later with the response of this tissue to graded doses of insulin. In Fig. 2, for example, the rate of gas evolution for the piece of tissue in the medium where the glucose concentration is 4 mg. per ml. was −3.6 μl. per 100 mg. of tissue per hour before insulin addition and +67.6 μl. after insulin addition. Thus the net change in gas evolution due to the addition of insulin is 67.6 μl. + 3.6 μl., or 71.2 μl. The corresponding net change for the tissue suspended in the medium where the glucose concentration is 1 mg. per ml. was 55.5 + 6.5, or 62.0 μl. per 100 mg. of tissue per hour. In Table I data are presented on the net changes observed in seven other experiments in which the rate in the presence of 1 mg. per ml. of glucose is compared with that in the presence of 4 mg. per ml. On the average the rate in the presence of 4 mg. per ml. of glucose is always higher than with 1 mg. per ml. The greatest difference is observed in those tissues which show very high activity. In this regard it should be noted that considerable variation in the tissue response is observed from animal to animal. One of the factors governing this response is the age of the animal, but there are indications that other unknown factors are also involved. At times we have encountered animals in which the epididymal fat pad has shown a marked positive gas exchange without the addition of insulin. In such cases the addition of insulin may cause but a very small further increase in the rate of gas evolution. We have sought to correlate such positive responses with the emotional or environmental conditions of the animal, but so far with little success.

As a result of such studies we have chosen to employ glucose at a concentration level of 3 mg. per ml. in the remainder of the experiments to be described in this paper. This concentration of glucose appears to be sufficient to allow the reaction to proceed at or very near the maximal rate and to maintain a linear rate for the duration of the experiments.

In the results presented so far the amount of insulin used has been such as to yield a final concentration in the flask of 10^5 micro units per ml., which, as will be seen, is more than sufficient to evoke a maximal response by the tissue. Since the sample of insulin employed contained 25 units per mg., this is equivalent to 0.004 mg. per ml. Naturally, one of the earliest questions we sought to answer was how small an amount of insulin would yield a response. We soon found that it was possible to lower the insulin concentration to 10^3 micro units per ml. and to obtain fairly consistently responses which were nearly equal to those given by 10^5 micro units per ml. In Table II the results of five such experiments are shown. In each of the experiments we have compared the rate of paired tissues from one rat and we have employed glucose at a concentration of 3 mg. per ml. It will be seen that on the average the rate with 10^3 micro units of insulin is 95 per cent of that obtained with 100 times this amount. This amount of difference in rate can not be considered significant since the variation in the maximal rate of different pieces of tissue from the same rat can be of this order of magnitude or larger. However, the important point

TABLE II

Effect of insulin concentration on rate of CO_2 production

Glucose concentration 3 mg. per ml.

Experiment	Insulin			Maximal response*
	Micro units per ml.			
	10^5	10^3	10^1	
	μl. CO_2/100 mg./hr.†			%
1	19.8	19.5		99
2	23.6	22.4		95
3	14.3	12.8		90
4	29.3	26.3		90
5	47.0	47.0		100
6	21.8		7.4	34
7	15.7		0	0
8	14.5		0	0
9	22.7		3.4	15
10	22.8		0	0

* The percentage of maximal response has been calculated in the manner described in the text for the data given in Fig. 3.

† Expressed as net change in gas evolution that occurs before and after addition of insulin.

which we wish to make at this time is that when we attempted to lower the amount of insulin to 10^2 micro units per ml., we obtained erratic and inconsistent results. In Table II the results of five experiments are given in which 10^2 micro units per ml. of insulin were employed. It will be seen that in three of the five experiments no response at all was obtained. Now it should be noted that up to this time we had been employing the Krebs-Ringer bicarbonate solution for both an incubation medium and the dilution of our insulin samples. We therefore turned our attention to the possibility that in the dilution of our insulin samples losses occurred either due to denaturation or adsorption on the glassware. In the method of insulin assay developed by Martin *et al.* (6), these workers regularly employed a gelatin containing Krebs-Ringer bicarbonate medium for dilution of their standard insulin samples as a precautionary measure against such losses. The importance of the presence of a substance such as gelatin in the medium used for dilution of the insulin samples and even in the incubation medium itself is shown by the results given in Figs. 3 and 4.

In Fig. 3 the results of an experiment are shown where a comparison is made of the results to be obtained when the insulin was diluted on the one hand with Krebs-Ringer bicarbonate and on the other with the same medium to which gelatin had been added. In each case the incubation medium was Krebs-Ringer bicarbonate. The medium which contained gelatin was prepared by adding 200 mg. of gelatin to 100 ml. of Krebs-Ringer bicarbonate solution contained in a stoppered graduated cylinder. The contents of the cylinder were mixed for several minutes, the undissolved gelatin allowed to settle to the bottom, and the supernatant fluid used for the experiment. Paired pieces of tissue from one rat were employed. The results are plotted in Fig. 3 in terms of the gas exchange per 100 mg. of tissue. After an initial period of 50 minutes, during which the usual negative pressure response was obtained, the side arm contents were added. No response was obtained in that flask (*A*) where the insulin had been diluted with the plain Krebs-Ringer bicarbonate medium. However, in the other flask (*B*) where the insulin had been diluted with the gelatin containing medium a definite response was obtained. In both cases the addition of the contents of the side arm should have resulted in a calculated concentration of insulin in the flask of 10^2 micro units per ml. At the end of 120 minutes both flasks were removed from their manometers and to the contents of each flask there was added 0.05 ml. of an insulin solution in plain Krebs-Ringer bicarbonate of such strength as to yield a final concentration of 10^5 micro units per ml. The flasks were then replaced on their respective manometers, returned to the water bath, and regassed. Readings were recommenced, as shown in Fig. 3, after a total interval of 20 minutes. It will be seen that by this time a marked positive pressure change was under way in both flasks and of about equal magnitude. Alongside each line on this graph are figures which express the rates of the reaction in terms of μl. of gas per 100 mg. of tissue per hour. Thus in the manner described previously the net changes in gas pressure in each flask may be calculated. In the case of flask A the net change between the initial period without insulin and the final period where excess insulin was present amounts to 43.0 + 4.9, or 47.9. The effect of tipping insulin from the side arm was 0. In the other flask, B, the corresponding net change from the initial to the final period is 41.1 + 7.0, or 48.1. Here, however, tipping the contents of the side arm caused a net change in gas exchange which is 11.2 + 7.0, or 18.2. It is now possible to express the response

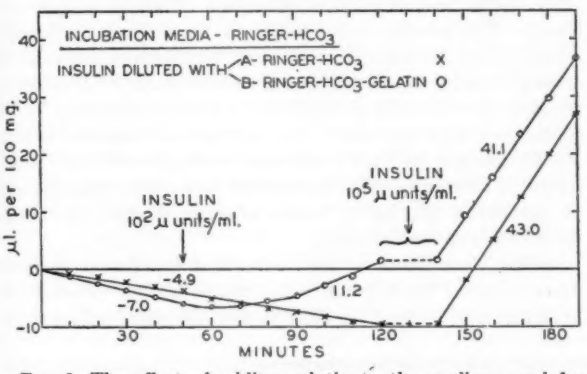

FIG. 3. The effect of adding gelatin to the medium used for dilution of the insulin samples. The main compartment of each flask contained 1.35 ml. of Krebs-Ringer bicarbonate to which glucose had been added to yield a final concentration of 3 mg. per ml. At 50 minutes 0.05 ml. of a solution of insulin calculated to yield a final concentration of 10^2 micro units per ml. was tipped from the side arm. In flask *A* the insulin solution was prepared by dilution with Krebs-Ringer bicarbonate glucose medium, while in flask *B* it was prepared by dilution with a Krebs-Ringer bicarbonate glucose medium which contained gelatin (see text). Dilutions were performed by the addition of 0.1 ml. of the working solution of insulin, which contained 3 units per ml. (see methods) to a 10-ml. stoppered graduated cylinder, followed by the addition of the appropriate medium to volume. One ml. of this solution was then further diluted in the same fashion to 10 ml. The amount of tissue added per flask was assumed to contribute 0.1 ml. to the total fluid volume in calculating the final insulin concentration in the flask. At the end of 120 minutes the experiment was temporarily stopped to permit addition of 0.05 ml. of an insulin solution directly to the flask contents so as to yield a concentration of 10^5 micro units per ml. in excess of that already present. The flasks were remounted on the manometers, regassed, and readings resumed at the time indicated. Paired tissues from a rat weighing 220 gm. were employed; the tissue in flask *A* weighed 300 mg., that in flask *B*, 279 mg. The numbers alongside each line give the rate in μl. per 100 mg. per hr.

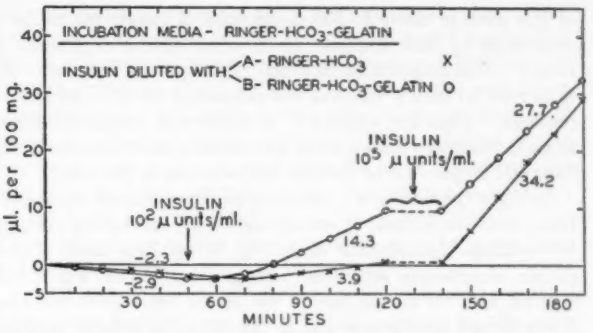

FIG. 4. The effect of adding gelatin to the medium used for incubation and for dilution of the insulin samples. The procedure employed was the same as that described for the experiment shown in Fig. 3 except that the medium added to the main compartment of the flask was a Krebs-Ringer bicarbonate glucose solution to which gelatin had been added. In flask *A* the insulin solution was prepared by dilution with Krebs-Ringer bicarbonate glucose medium, while in flask *B* it was prepared by dilution with the gelatin containing medium. Paired tissues were employed. The tissue in flask *A* weighed 286 mg. and that in flask *B* 246 mg.

of this piece of tissue to 100 micro units of insulin per ml. as a percentage of that response given when insulin is present in excess. This amounts to 18.2/48.1 × 100, or 38 per cent. We shall refer to such a value as the percentage of maximal insulin response. Thus the addition of an amount of insulin calculated to yield 100 micro units per ml. has caused a zero response in one flask and 38 per cent of the maximal response in the other.

In Fig. 4 the results of a similar type of experiment are shown. Here, however, instead of employing for the incubation medium Krebs-Ringer bicarbonate as in Fig. 3, we have used Krebs-Ringer bicarbonate saturated with gelatin. As in Fig. 3 the medium used for the dilution of the insulin has been in one case Krebs-Ringer bicarbonate and in the other the gelatin containing medium. In this experiment it can be seen that when insulin is tipped from the side arm so as to yield a calculated concentration of 100 micro units per ml. in the flask a response is obtained in both cases. The response, however, obtained in the flask where the insulin was diluted with plain Krebs-Ringer bicarbonate is much less than when the gelatin-saturated medium was employed for dilution. Here again, as in the experiment of Fig. 3, the flasks were removed at the end of 120 minutes to permit the addition of 0.05 ml. of an insulin solution so as to yield a concentration of 10^5 micro units per ml. in each flask. Again both tissues responded with a more rapid outpouring of carbon dioxide of nearly equal magnitude per 100 mg. of wet weight. Here the percentages of the maximal insulin response calculated as described above for the two cases are 55 and 18 per cent, respectively.

From these experiments, which are representative of others that have been performed, it is clear that when working with dilute solutions of insulin in simple salt solutions, losses in activity of considerable magnitude may take place. This may occur not only during the procedure used for dilution of the insulin sample, but also after addition of the diluted solution to the incubation flask. It is therefore important in such experiments to use media containing gelatin, or perhaps some other protein, both for serial dilution of the insulin samples and as the fluid in which the tissue is to be incubated. Such precautions would not appear to be necessary when an excess of insulin is added, as for example when the final concentration in the incubation medium is of the order of 10^5 micro units per ml. Indeed the results presented in Table II would indicate that the sensitivity of the epididymal fat pad is such that the effect of any such losses would be scarcely detectable even when the final concentration of insulin in the incubation flask reaches a value $\frac{1}{100}$ of this (10^3 micro units per ml.). In one experiment, however, which was run exactly like that presented in Fig. 3 except that 500 micro units were employed instead of 100, a higher response was obtained when the insulin was diluted with a gelatin containing medium than with one without it. The percentages of maximal response obtained were 76 and 61, respectively. It would therefore appear that the safest procedure, until proved otherwise, would be to use gelatin-containing media throughout when working with final insulin concentrations of 10^3 micro units per ml. or lower, and under the experimental conditions employed here. Our routine procedure now, when working with insulin concentrations in this range, is to prepare media fresh daily by adding Krebs-Ringer bicarbonate to the appropriate weighed amounts of solid gelatin and glucose. Media containing gelatin should not be stored overnight in the cold since we have found that this gives rise to hazy solutions which yield low and erratic results.

We have no information as to the cause of the loss of insulin activity that occurs in the absence of gelatin. We surmise that it is due, in part at least, to adsorption of insulin on glass surfaces. This premise is supported by the findings of Ferrebee *et al.* (7) and Narahara *et al.* (8) who, by means of radioactivity measurements, showed that insulin labeled with I^{131} is adsorbed to glassware and that this adsorption may be prevented by the presence of serum albumin or by an excess of unlabeled insulin. At the moment we have no information on the effect of the addition of substances other than gelatin as a means of minimizing or preventing this loss of insulin activity, though experiments toward this end are planned.

In Fig. 5 are presented the results of an experiment where we have measured the response to be obtained at four different concentrations of insulin; 25, 50, 100, and 250 micro units per ml.

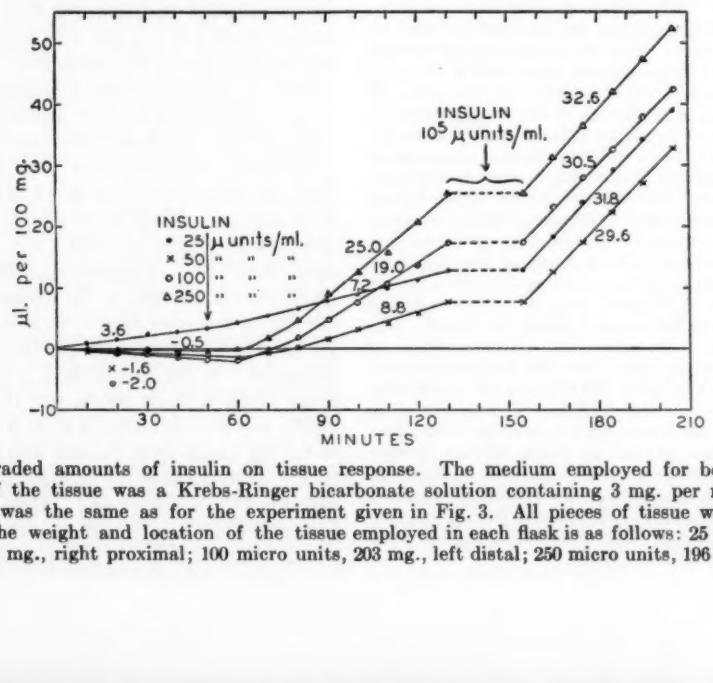

FIG. 5. The effect of graded amounts of insulin on tissue response. The medium employed for both dilution of the insulin samples and incubation of the tissue was a Krebs-Ringer bicarbonate solution containing 3 mg. per ml. of glucose and gelatin. Otherwise the procedure was the same as for the experiment given in Fig. 3. All pieces of tissue were obtained from a single rat (weight 219 gm.). The weight and location of the tissue employed in each flask is as follows: 25 micro units, 208 mg., right distal; 50 micro units, 193 mg., right proximal; 100 micro units, 203 mg., left distal; 250 micro units, 196 mg., left proximal.

The results are plotted in terms of response per 100 mg. of tissue. Four pieces of tissue from one rat were employed in this experiment and the procedure followed was similar to that outlined for the experiments given in Figs. 3 and 4. Here, however, a gelatin medium was employed throughout for both dilution of the insulin samples and as incubation fluid. It is clearly evident that the response obtained upon tipping in the insulin from the side arm at 50 minutes is a function of the amount of insulin added. It should be noted that at concentrations of insulin within this range a period of 30 minutes or so may be required before a linear rate is established after the addition of the insulin samples. Thus the rate values given on Fig. 5 for this part of the experiment refer to that portion of the curve where a linear rate has been achieved. A calculation of the percentage of maximal insulin response by the procedure outlined above yields the following values: 25 micro units per ml., 13 per cent; 50 micro units per ml., 33 per cent; 100 micro units per ml., 65 per cent; and 250 micro units per ml., 76 per cent.

The data presented in Fig. 5 serve to illustrate the fact that on occasion the initial response shown by one of four pieces of tissue from the same rat may show a slight positive response rather than the more usual negative one. In the experiment shown here it so happened that this occurred in the flask which had been set up to contain the lowest concentration of insulin. Here then is a good example of the reasons why we have chosen to express the response to added insulin in terms of the net change rather than in terms of the absolute rate. In this experiment there is very little difference between the absolute rates for 25 and 50 micro units of added insulin, but an appreciable difference in terms of the net change. The only criterion we can offer in justification for this adopted procedure of calculation is the fact that in an aberrant case such as the one shown here the result obtained agrees best with the mean of other experiments where the initial tissue response was not aberrant. For example, the mean value for the percentage of maximal insulin response obtained at 25 micro units per ml. of insulin in four experiments is 15, as compared to 13 for the experiment shown in Fig. 5.

It is obvious that an experimental procedure of the sort employed in obtaining the data presented in Fig. 5. offers interesting possibilities for the rapid and quick assay of insulin. In such a technique each piece of tissue can serve as its own control when results are expressed in terms of the percentage of maximal insulin response. Indeed, it is not inconceivable that a standard dose response curve might be established for this tissue from which at least approximate values of the insulin content of unknown samples could be read. For example, to date we have run 14 experiments at insulin concentrations of 50 micro units per ml. These yield a mean value of 25 for the percentage of maximum insulin response, with a range of 16 to 34. In six experiments at 100 micro units per ml. the corresponding mean value is 61, with a range of 51 to 69 per cent. Preliminary experiments have shown that the experimental procedure is applicable to plasma. It would appear, therefore, that the method might be suitable for the rapid and simple assay of insulin-like activity in plasma or serum samples, and experiments toward this end are planned.

DISCUSSION

Though no positive proof has been presented, it seems reasonable to assume that the positive gas pressures generated by this tissue on addition of insulin are due to carbon dioxide. From the results of Winegrad and Renold (1, 2) it would appear that most of this carbon dioxide is generated by way of the reactions given in Equations 1 and 2. Some carbon dioxide may be released from the bicarbonate of the medium due to lactic acid formation. The percentage of the total that is formed in this manner must be relatively small since the lactic acid determinations that we have performed so far have shown that on the average lactic acid production can at best account for only 10 to 20 per cent of the carbon dioxide released when an active piece of tissue is employed.

It is of interest to compare the sensitivity of the adipose tissue to insulin as observed here with a few of the many reports in the literature on the sensitivity of other tissues to insulin. There is a wide discrepancy in the reported sensitivity of the rat diaphragm to insulin. Some workers, such as Stadie and Zapp (9) and Perlmutter *et al.* (10) were unable to obtain any increase in glycogen synthesis by the rat diaphragm when the insulin concentration fell below 10^{-3} units per ml. A definite effect on glucose utilization by the diaphragm was reported by Krahl and Park (11) at 10^{-4} units of insulin per ml. with a marginal effect at 10^{-5} units per ml. On the other hand Willebrands *et al.* (12) obtained responses on the rat diaphragm with as low as 10^{-5} to 10^{-6} units per ml. A perusal of the papers of these various authors has failed to reveal any information concerning the mode of dilution of insulin samples which they employed. Randle (13) in his studies on glucose uptake by the rat diaphragm clearly states that dilution of his insulin samples was carried out with the same inorganic medium employed for incubation of the tissue. He reports that the minimal detectable concentration of insulin varied from experiment to experiment, the range of values being of the order of 10^{-4} to 2×10^{-3} units per ml. These findings are similar to those presented here for adipose tissue when a plain Krebs-Ringer bicarbonate solution was employed for both dilution of the insulin samples and incubation medium (Table II). Balmain *et al.* (14) with the use of the same manometric technique employed here found that slices of mammary gland were unresponsive to insulin concentrations below 10^{-3} units per ml. In considering these reports by other workers one wonders whether a loss of insulin activity at low concentrations such as we have encountered might account in part for their discordant results. It would be of interest to know whether the rat diaphragm would show a greater sensitivity and more consistent responses to insulin if a gelatin containing medium were to be employed both for dilution of insulin samples and for the incubation of the tissue.

The results that we have obtained to date would indicate that the epididymal fat pad gives graded responses to insulin over a concentration range of 10^{-5} to 10^{-3} units per ml. The concentration at which the response is approximately 50 per cent of the maximum appears to be a little below 10^{-4} units per ml. These parameters are in general agreement with those reported by Willebrands and Groen (15) for the effect of insulin upon the rat diaphragm. It is also of interest to note in this connection that the concentration of insulin reported by various workers for normal serum or plasma from several animals, including man, is on the average 10^{-4} units per ml. (*cf.* Willebrands and Groen (15)). Thus the insulin responses observed here on adipose tissue *in vitro* would appear to be within the physiological range of concentrations for this hormone.

SUMMARY

A simple and rapid procedure for following the action of insulin *in vitro* upon the rat epididymal fat pad is described. It is based upon the measurement of the time course of the over-all gas pressure changes as observed in a Warburg vessel in the absence of a CO_2 absorbent. The metabolism of this adipose tissue in the presence or absence of glucose and without insulin is such that more gas is absorbed than is released, i.e. the R. Q. is less than 1. Upon the addition of insulin and in the presence of glucose a rapid change in metabolism occurs which is reflected in the total gas exchange. The amount of gas released now greatly exceeds the gas consumption. This is presumably due to the release of CO_2 which occurs during the synthesis of fat from glucose. The rate of gas release is dependent upon the glucose concentration of the medium and is linear with time in the presence of ample glucose supplies. The rate of gas release is also a function of the insulin concentration within the range 10^{-5} to 10^{-3} units per ml. It is shown that when concentrations of insulin within this range are employed in simple salt solutions, unexplained losses of insulin activity occur. These may be prevented by the addition of gelatin to the inorganic media used both for dilution of the insulin samples and for the incubation fluid.

REFERENCES

1. Winegrad, A. I., and Renold, A. E., *J. Biol. Chem.*, **233**, 267 (1958).
2. Winegrad, A. I., and Renold, A. E., *J. Biol. Chem.*, **233**, 273 (1958).
3. Balmain, J. H., French, T. H., and Folley, S. J., *Nature*, **165**, 807 (1950).
4. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
5. Haugaard, N., and Marsh, J. B., *J. Biol. Chem.*, **194**, 33 (1952).
6. Martin, D. B., Renold, A. E., and Dagenais, Y. M., *Lancet*, **2**, 76 (1958).
7. Ferrebee, J. W., Johnson, B. B., Mithoefer, J. C., and Gardella, J. W., *Endocrinology*, **48**, 277 (1951).
8. Nabahara, H. T., Tomizawa, H. H., Miller, R., and Williams, R. H., *J. Biol. Chem.*, **217**, 675 (1955).
9. Stadie, W. C., and Zapp, J. A., Jr., *J. Biol. Chem.*, **170**, 55 (1947).
10. Perlmutter, M., Weisenfeld, S., and Mufson, M., *Endocrinology*, **50**, 442 (1952).
11. Krahl, M. E., and Park, C. R., *J. Biol. Chem.*, **174**, 939 (1948).
12. Willebrands, A. F., Groen, J., Kamminga, C. E., and Blickman, J. R., *Science*, **112**, 277 (1950).
13. Randle, P. J., *J. Endocrinol.*, **14**, 82 (1956–1957).
14. Balmain, J. H., Cox, C. P., Folley, S. J., and McNaught, M. L., *J. Endocrinol.*, **11**, 269 (1954).
15. Willebrands, A. F., and Groen, J., *Advances in Internal Med.*, **6**, 331 (1954).

Studies on the Metabolism of Adipose Tissue

II. THE EFFECT OF CHANGES IN THE IONIC COMPOSITION OF THE MEDIUM UPON THE RESPONSE TO INSULIN*

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In the first paper of this series (1) it was shown that the addition of insulin *in vitro* to the epididymal fat pad of the rat produces a change in the pad's metabolism which was reflected in a marked increase in the R. Q. of the tissue which could be readily measured manometrically. On the basis of the work of Winegrad and Renold (2, 3) and the fact that a concomitant increase in O_2 consumption is not observed, this marked alteration in the overall gas exchange was considered to be an indication of the stimulation by insulin of fatty acid synthesis from glucose. In the experiments to be reported here a study has been made of the effect of alterations in the composition of the medium on this response of the epididymal fat pad to insulin. This investigation was undertaken chiefly for the purpose of ascertaining the optimal conditions to be employed before proceeding to further studies on this tissue. Incidental to this study it was necessary to make a comparison of the response shown under uniform conditions by each of four pieces of tissue obtained from the epididymal fat pads of a single rat. Thus data on this point are also presented along with observations on a comparison of wet weight versus total nitrogen values as a basis for the expression of tissue activity. Some results on the variation in adipose tissue response to insulin in relation to the age of the rat are also given.

EXPERIMENTAL

Male rats obtained from the Holtzman Company and maintained on a diet of Purina laboratory chow *ad libitum* were used throughout. The procedure for obtaining the epididymal tissue and its handling were in general as described previously (1). Usually in this study two pieces of tissue were obtained from the right epididymal fat pad and two from the left in the following fashion. The free portion of the fat pad was removed from the animal by holding it with forceps just distal to the point of entry of the epididymal blood vessels and cutting at this level. A piece of tissue (100 to 200 mg.) was now cut off the free tip of the fat pad; this is called the distal portion. The piece of tissue next to the distal portion (100 to 200 mg.) was then cut off; this is called the proximal portion. That part of the fat pad which had been held with the forceps was discarded.

Warburg vessels without a center well and with a total volume of 8 to 12 ml. were employed. In the main compartment of the vessels there were placed 1.35 ml. of an incubation medium which contained 3.6 mg. of glucose per ml. The side arm contained 0.05 ml. of an insulin solution (3 units per ml.). Upon tipping the contents of the side arm the final concentration of insulin in the flask thus becomes 0.1 unit per ml. if it is assumed that the piece of tissue contributes 0.1 ml. to the total volume. This concentration of insulin has been found to be approximately 100 times that necessary to cause maximal stimulation (1). The stock solution of insulin was prepared from the same insulin sample and in the same manner as described previously (1). Working solutions containing 3 units per ml. were prepared by diluting this stock solution with the medium to be employed for incubation.

In all cases the CO_2 gas mixture to be employed was bubbled through the medium to adjust the pH before the addition of the tissue. After attachment of the flasks to their manometers the flask contents were equilibrated with this gas mixture for 5 minutes at 37°. Unless otherwise stated the gas mixture employed was 5 per cent CO_2–95 per cent air.

We have employed the bicarbonate-buffered Ringer solution described by Krebs and Henseleit (4) as a standard with which to compare media of different composition. This medium (No. 1, Fig. 2) was modified by alterations in its composition in the manner shown in Fig. 2. In addition, three other incubation media were used in which the cation was either all sodium, all potassium, or an equal mixture of the two and the only anions were chloride and bicarbonate. These media had the following composition: *A*. NaCl, 0.125 M; $NaHCO_3$, 0.025 M; *B*. KCl, 0.129 M; $KHCO_3$, 0.025 M; *C*. NaCl, 0.063 M; KCl, 0.065 M; $NaHCO_3$, 0.0125 M; $KHCO_3$, 0.0125 M.

In some experiments the tissues were removed from the flasks at the end of the incubation and prepared for nitrogen estimations. They were homogenized in either (*a*) 10 per cent trichloroacetic acid or (*b*) a mixture of alcohol (45 per cent)-ether (25 per cent)-water (30 per cent) and made to a total volume of 10 ml. with the same fluid used to make the homogenate. An aliquot (usually 0.5 ml.) of this homogenate was digested with 1 ml. of 9 N sulfuric acid; after approximately 1.5 hours of heating 3 drops of 20 per cent hydrogen peroxide were added and the sample was reheated for 15 minutes. The ammonium ion formed was measured colorimetrically with the use of Nessler's reagent. Homogenization of the tissue in alcohol-ether-water mixture, as recommended by Aldridge (5) for precipitating protein, has an advantage over the use of water or trichloroacetic acid in that the fat dissolves or emulsifies in the organic solvents and does not adhere to the walls of the glassware thus entrapping protein with it. The accuracy of the pipetting is therefore greater when this medium is used.

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The data to be presented were obtained with the same general experimental procedure described previously (1). Manometric readings were made on each piece of tissue at 10-minute intervals for a 40- to 60-minute initial period before tipping insulin from the side arm. Readings were then continued in the same fashion for a period of time sufficient to establish the linearity of the rate, usually 40 to 60 minutes. Results are expressed in terms of μl. of CO_2 evolved per hour per 100 mg. of tissue (wet weight) as calculated from the net change in rate that occurs before and after the addition of insulin.

RESULTS

The general procedure employed in these studies is one in which four pieces of adipose tissue from a single rat are compared one with another under conditions where the composition of the medium or gas phase is varied. It is therefore important to establish first the degree of variability that may be expected when the responses of four pieces of tissue from one rat are compared under identical experimental conditions. In Table I there are presented the results obtained of such a comparison in 25 experiments. Two points are brought out by these results. First, there is a variation in the response obtained in different pieces of tissue from the same rat. In an individual experiment this variation may be fairly large, as for example in Experiments 3, 14, 20, and 22 where the rate for one piece deviates from the mean for all four by 28 to 50 per cent. In other cases there may be very good agreement of the rates for all four pieces of tissue, *e.g.* Experiments 7, 19, and 23. However, if the mean values for the 25 experiments shown in Table I are compared and the value for the right distal portion of tissue is taken as 100, then the greatest variation observed in the series is no more than 12 per cent. It would appear that on the average the left fat pad shows more activity than the right and that the distal portion is more active than the proximal. The latter result may be a reflection of the fact that the distal portion of the fat pad is much the thinner of the two. It is also of interest to note that in those experiments (3, 14, 20, 22) where the poorest agreement is found, the piece of tissue which is most out of line is the left distal portion.

This variability in the activity as expressed in terms of total wet weight for different pieces of adipose tissue from the same rat is perhaps not too surprising when it is considered that the cellular protoplasm of this tissue forms but a small portion of the total wet weight. The total nitrogen content of this tissue is on the average only 0.26 mg. per 100 mg. wet weight. It was therefore of interest to learn whether better agreement would be obtained if activities were expressed in terms of total tissue nitrogen rather than on a wet weight basis. The results of eight experiments directed toward this end are given in Table II. In each of these experiments four pieces of tissue from a single rat were weighed and their maximal response in the presence of 0.1 unit of insulin per ml. was measured in the usual manner. At the end of the incubation the tissues were removed from the Warburg vessels and homogenized. The incubation medium was discarded. In the first four experiments listed in Table II 10 per cent trichloroacetic acid was used as the fluid for homogenization; in the second four an alcohol-ether-water mixture was

TABLE I

Comparison of responses to insulin of four pieces of epididymal adipose tissue from one rat

Each experiment was performed in the same manner as that depicted in Fig. 3 except that the incubation medium for all four pieces of tissue was Krebs-Ringer bicarbonate solution; gas phase, 95 per cent air-5 per cent CO_2; insulin concentration 0.1 unit per ml.

Experiment	Tissue			
	Right distal	Right proximal	Left distal	Left proximal
	μl. CO_2/100 mg./hr.			
1	39.8	61.5	55.0	54.5
2	29.3	33.2	29.5	23.6
3	18.4	14.0	30.0	20.8
4	26.4	29.4	23.7	23.6
5	28.4	23.4	26.7	31.0
6	69.1	63.1	87.5	63.5
7	31.8	29.6	30.5	32.6
8	31.1	27.8	39.8	34.6
9	29.0	31.2	22.4	20.4
10	20.4	20.8	22.7	29.3
11	13.4	22.8	16.2	24.4
12	8.4	9.1	10.3	9.6
13	35.4	27.3	31.0	39.0
14	47.2	41.3	74.4	42.9
15	61.0	47.3	50.3	44.5
16	48.5	36.1	50.0	31.2
17	59.3	44.3	56.3	55.0
18	16.6	20.5	23.1	23.6
19	21.3	19.4	21.8	17.4
20	30.2	15.7	46.9	34.1
21	22.0	17.0	19.4	17.1
22	25.8	22.6	39.5	32.5
23	30.9	33.7	36.4	33.2
24	32.7	16.9	30.5	23.8
25	25.7	15.9	24.4	18.1
Mean	32.1	29.0	35.9	31.2
Per cent	100	90.5	112	97.5

TABLE II

Comparison of activity of tissue as expressed in terms of its wet weight versus its total nitrogen content

In the preparation of the extracts for nitrogen estimations the tissue was homogenized in 10 per cent trichloroacetic acid in Experiments 1–4 and in an alcohol(45 per cent)-ether(25 per cent)-water(30 per cent) mixture in Experiments 5–8. All other conditions as described for the data presented in Table I.

Experiment	Wet weight				Total nitrogen			
	Right distal	Right proximal	Left distal	Left proximal	Right distal	Right proximal	Left distal	Left proximal
	μl. CO_2/100 mg. tissue/hr.				*μl. CO_2/100 μg. N/hr.*			
1	16.6	20.5	23.1	23.6	6.5	5.9	9.2	5.9
2	21.3	19.4	21.8	17.4	8.5	7.1	11.1	9.2
3	30.2	15.7	46.9	34.1	13.1	5.8	19.1	17.3
4	22.0	17.0	19.4	17.1	8.3	6.2	19.5	7.0
5	26.2	23.9	41.2	34.0	12.3	10.7	22.8	16.6
6	30.9	33.7	36.4	33.2	18.1	29.2	23.1	27.0
7	32.7	16.9	30.5	23.8	16.2	11.0	13.3	13.6
8	25.7	15.9	24.4	18.1	52.5	64.5	40.7	37.5
Mean	25.7	20.4	30.5	25.2	16.2	16.8	19.8	16.0
Per cent	100	80	119	98	100	104	122	99

employed. Total nitrogen was then determined as described under "Experimental." No attempt has been made to correct for the nitrogen contributed by the added insulin as this is insignificant (1 μg. of N per flask).

It can be seen from the data presented that the use of total nitrogen values as a basis for the calculation of activity did not appreciably alter the variation in activity encountered in the four pieces of tissue obtained from one rat. In general the pattern of variation runs the same regardless of the means used for expression of the results. For some unknown reason the nitrogen content of the tissue used in Experiment 8 was unusually low. It would seem therefore that for the purposes at hand the wet weight of the tissue provides as satisfactory a basis for the calculation of results as total nitrogen.

In preliminary experiments attempts were made to estimate the protein N of the adipose tissue. When trichloroacetic acid was used as the precipitating agent only 5 to 15 per cent of the total N could be centrifuged down as protein N; the remainder was trapped in the supernatant fatty layer. When alcohol-ether-water mixture was used as the homogenizing medium, from 50 to 96 per cent of the total N could be centrifuged down. However, the proportion of total N to protein N was never sufficiently constant for us to place confidence in the use of the values as a basis for expressing activity.

The second point that is brought out by the data of Table II is that the magnitude of the response obtained varies from rat to rat. This is due in part to the age of the rat, as is shown by the data presented in Fig. 1 where the maximal response obtained to insulin in terms of μl. of CO_2 per 100 mg. per hr. is plotted against the weight of the rat from which the tissue was obtained. Each point on this figure represents the rate observed for the right distal portion of the epididymal fat pad of one rat, a total of 87 rats being represented all told. The greatest response to insulin is shown by tissue from rats with weights in the range of 160 to 250 gm. Tissue obtained from rats whose weight exceeds 250 gm. shows a much smaller response to insulin and the values tend to form a plateau at a value in the neighborhood of 10 μl. for the older rats. Preliminary experiments indicate that the small amount of CO_2 production evoked by insulin in tissue from the older rats can largely be accounted for by lactic acid production.

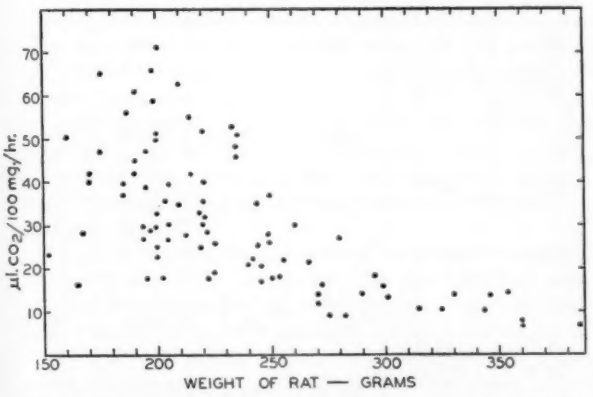

FIG. 1. The response of epididymal adipose tissue to insulin in relation to the weight of the rat from which it was obtained. Each point represents the rate for the right distal portion of the epididymal fat pad in response to insulin, 0.1 unit per ml. Experimental conditions as described in the text.

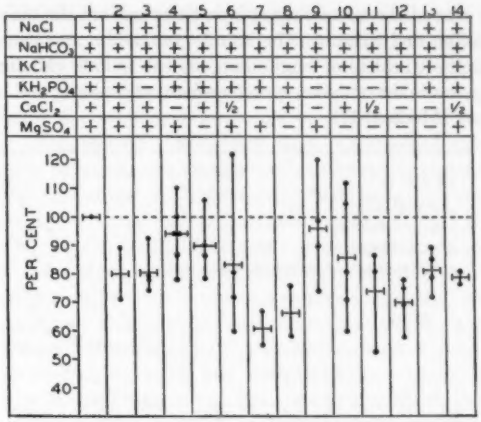

FIG. 2. The effect of changes in the inorganic composition of the Krebs-Ringer incubation medium upon the response of epididymal adipose tissue to insulin. The standard medium (No. 1) is made by combining the following: 100 ml. of 0.154 M NaCl, 21 ml. of 0.154 M $NaHCO_3$, 4 ml. of 0.154 M KCl, 1 ml. of 0.154 M KH_2PO_4, 3 ml. of 0.11 M $CaCl_2$, 1 ml. of 0.154 M $MgSO_4$. The presence or absence of a component of medium No. 1 is indicated by a plus or minus sign while the figure ½ means that the concentration is one-half that present in medium No. 1. When a component was omitted, water was added in its place so as to bring the medium to the same total volume. Each experiment is represented by ● and the mean for any given set of experiments is indicated by a horizontal line. In each case the response obtained in an experimental medium is compared with the response shown by a piece of tissue from the same rat in medium No. 1. The response obtained in medium No. 1 is taken as unity and the response in the experimental medium is expressed in terms of the percentage of this value. Other experimental conditions as described in the text.

As a consequence of these observations we now employ rats in the weight range of 150 to 250 gm. for most of our experiments.

In Fig. 2 data are presented on the effect of changes in the composition of the Krebs-Ringer bicarbonate solution on the response of the adipose tissue to insulin. The result of each experiment is represented by a solid circle and expressed in terms of percentage of response obtained with a piece of tissue from the same rat in Krebs-Ringer-bicarbonate (Medium 1). An effort was made to see that the piece of tissue employed in the standard medium was varied as to its source so that sometimes it was from the left fat pad, sometimes from the right, sometimes the distal portion and sometimes the proximal. The basic experimental procedure employed to obtain this data was similar to that in the experiment which is depicted in Fig. 3. The mean of the results for any given medium is indicated by a horizontal line.

The results shown in Fig. 2 indicate that alterations in the standard Krebs-Ringer bicarbonate medium produced in no case a better medium for tissue activity, the mean lying always below 100 per cent. In interpreting these results, however, we feel that little significance should be attached to any experiment in which the mean lies within about 80 per cent of the response of the standard medium. This is because variations of about this magnitude may be encountered in a series of experiments between pieces of tissue from the same rat even in identical media as is shown by the data of Table I. Hence one may conclude that very little effect is to be noted if certain single components or even certain pairs of components of the standard medium are omitted. The only modifications which can be considered to

have altered the tissue response on this basis are the omission of potassium and calcium (Medium 7); potassium and magnesium (Medium 8); calcium, magnesium, and phosphate (Medium 12); and perhaps the omission of magnesium, phosphate, and half the calcium (Medium 11).

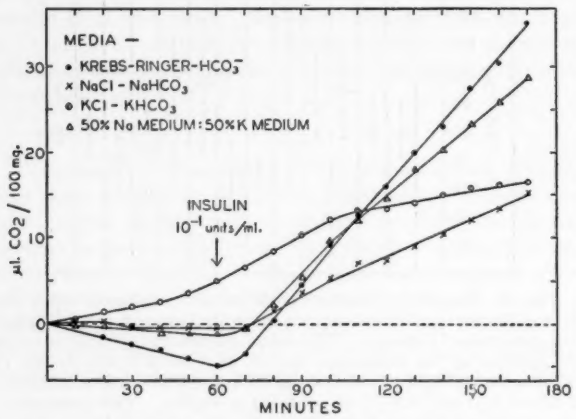

FIG. 3. The response of epididymal adipose tissue to insulin in a medium containing either sodium or potassium as the single cation. Four pieces of tissue from a single rat were employed. The composition of the media is given in the text. Gas phase 5 per cent CO_2-95 per cent air; glucose concentration, 3.6 mg. per ml.; insulin added at 60 minutes to yield a final concentration in the flask of 0.1 unit per ml. Temp. 37.2°.

It should be pointed out that a small dilution of not more than 5 per cent occurred when some of the modified media were prepared. A few experiments were therefore run in which Krebs-Ringer-bicarbonate solution was diluted to this same extent and compared with the undiluted medium. No significant difference was found.

Experiments were next run in media which contained either potassium or sodium as the only cation and chloride and bicarbonate as the anions. The composition of these media are given in the text. A typical experiment of this sort is illustrated in Fig. 3. Here four pieces of tissue from a single rat were each run in a different medium. One piece was incubated in Krebs-Ringer bicarbonate which served as a standard for comparison. In this medium the tissue showed the usual metabolic pattern of an R. Q. lower than 1 before insulin is added and a marked evolution of CO_2 after insulin addition, which was linear with time. When the fat pad was incubated in the medium in which potassium was the only cation, its R. Q. was greater than 1 before the addition of insulin and the rate tended to increase with time. The addition of insulin caused but a little change in this rate and in time the rate decreased below the initial. In an all sodium medium the pattern of metabolism is more like that seen in the Krebs-Ringer bicarbonate medium. The initial R. Q. is near to 1 and the addition of insulin stimulates CO_2 production. The response to insulin is, however, never as great in the all sodium medium as in the Krebs-Ringer bicarbonate and the rate does not remain linear with time but tends to decrease as the experiment progresses. When the incubation medium is composed of

TABLE III

Tissue activity in relation to changes in pH of medium as produced by variations in bicarbonate concentration and CO_2 tension

Rates are expressed as μl. of CO_2 per 100 mg. of tissue (wet weight) per hour. The rate observed at pH 7.40 is used as the standard (100 per cent) in calculation of the percentage response. All experiments were run in a calcium free medium (see text for further details) and in the manner depicted for the experiment given in Fig. 3. The pH values are those determined with the glass electrode at approximately 25° on aliquots of the media equilibrated at this same temperature with the gas mixture employed. pH values were also determined on the incubation media at the end of the run and did not vary appreciably from those given. The gas mixtures employed were CO_2-air mixtures obtained commercially. The mixture reported as containing 5 per cent CO_2 was not analyzed and is therefore an approximate value.

pH	7.40		7.22		7.88		7.22		7.53	
CO_2 in gas phase	5%		5%		5%		9.2%		9.2%	
HCO_3^-, mmoles/l.	25		12.5		50		25		50	
	Rate	%	Rate	%	Rate	%	Rate	%	Rate	%
Rat No. 1	62.0		33.2	53.5	48.0	77.5				
			28.6	46.0						
2	48.0						28.0	58.0	44.9	93.5
									37.4	78.0
3	34.8				21.5	62.0				
4	47.7						29.5	61.8	52.9	111.0
									36.0	75.5
5	58.0		41.5	71.5	38.9	67.0				
			39.3	68.0						
6	21.0						17.1	81.5		
							17.1	81.5	19.9	95.0
7	29.5				19.7	72.0				
			21.2	77.0	30.3	110.0				
8	50.0						37.2	74.5		
							42.9	86.0	41.5	84.0
9	34.5		26.7	77.5	35.3	103.0				
Mean (%)		100		65.6		81.9		76.9		89.5

TABLE IV

Tissue activity in relation to oxygen content of gas phase

Each experiment performed in a manner similar to that shown in Fig. 3 with Krebs-Ringer bicarbonate medium and an insulin concentration of 0.1 unit per ml.

Experiment	Gas phase	
	5% CO_2-95% air	5% CO_2-95% O_2
	µl. CO_2/100 mg./hr.	
1	18.0	14.9
2	27.2	32.8
3	39.6	59.7
4	43.0	46.0
5	44.6	44.9
6	55.5	39.3
Mean	38.0	39.6

equal parts of the all sodium medium and the all potassium medium, the response of the tissue is always better than in either one of the two media alone and it approaches more nearly the behavior of tissue in Krebs-Ringer bicarbonate. This pattern has been seen repeatedly in a series of experiments of this type.

The results of studies of the effect of pH on the response of rat epididymal adipose tissue to insulin are shown in Table III. In these experiments the pH was varied as indicated by alterations in the bicarbonate concentration and the CO_2 tension. Four pieces of tissue from one rat were employed in each experiment; one piece in all cases being run in the standard medium as a basis for comparison. The standard medium employed here was the Krebs-Ringer bicarbonate from which calcium was omitted since at the higher concentration of bicarbonate a precipitate of calcium salts occurred. As shown by the data presented in Fig. 2 the omission of calcium from the medium does not itself cause any significant change in the response of the tissue to insulin. Alterations in the sodium bicarbonate content of the experimental media were compensated for by the addition or removal of equimolar amounts of NaCl so that the total osmolarity of the solution remained unchanged. The results obtained have been expressed in Table III in terms of the absolute rates and also as the percentage of the response obtained in the experimental media with that in the standard. On the average it will be seen that the rate falls off as the pH is varied on either side of 7.4. This occurs whether the change in pH is brought about by alteration of the bicarbonate concentration or CO_2 tension or both. The tissue would appear from these results to be more sensitive to changes on the acid side than on the alkaline side of pH 7.4. We conclude from these experiments that a pH value near 7.4 is optimum for the stimulation by insulin of lipogenesis in the epididymal fat pad.

Finally, a comparison has been made of the response to insulin by the rat epididymal fat pad when the gas phase was 5 per cent CO_2-95 per cent O_2, as compared with 5 per cent CO_2-95 per cent air. The results of six experiments of this type are shown in Table IV. On the average no important differences are to be noted by changes in the oxygen content of the gas phase.

DISCUSSION

The data presented here indicate that the stimulation by insulin *in vitro* of metabolic processes in the epididymal fat pad is sensitive to alterations in the inorganic composition of the incubation medium. The behavior of adipose tissue in this respect is not unlike that of the rat diaphragm on which studies of a similar nature have been made with regard to the ability of insulin to stimulate the conversion of glucose to glycogen in this tissue *in vitro*. For example, Stadie and Zapp (6) reported that the stimulation by insulin of glycogen synthesis in rat diaphragm was prevented by the use of a medium in which potassium was the only cation. A similar finding for mouse diaphragm was reported by Oyama and Grant (7). The results presented here show that the use of a medium containing potassium as the only cation results not only in a failure to obtain a response to insulin but produces an initial, though short lived, increase in the R. Q. of the adipose tissue. On the other hand, the omission of potassium from the medium has much less effect upon the response of adipose tissue or diaphragm to insulin. Stadie and Zapp (6) found that a maximal effect of insulin on glycogen synthesis in rat diaphragm *in vitro* was obtained in a phosphate-buffered saline solution in the complete absence of potassium. Adipose tissue responds to insulin in a medium containing sodium as the only cation, but the response is far better if a small amount of potassium or calcium and magnesium is present. Thus the metabolism of glucose by both these tissues in response to insulin appears to be more favorable in a medium whose cationic composition is similar to that of the extracellular fluids. This is not surprising and is in agreement with other studies made on the metabolism of glucose by intact pieces of tissue *in vitro*. For example, Ball *et al.* (8) have shown that the very high rate of conversion of glucose to lactic acid exhibited by the intact red gland of the fish swim bladder in an all sodium medium diminishes progressively as the ratio of potassium to sodium in the incubation medium increases. On the other hand, when dealing with tissue slices such as liver, the work of Flink *et al.* (9) and Hastings *et al.* (10) has shown that a high potassium environment is needed to maintain the normal high intracellular potassium content which seems to be necessary for the demonstration of glycogen synthesis from glucose.

The main variation in the anionic composition of the medium which has been studied here has been the omission of phosphate which would appear to have but little effect on the response of adipose tissue. Stadie and Zapp (6) have reported that the omission of phosphate from the medium is without effect upon the stimulation by insulin of glycogen synthesis in the rat diaphragm. It is important to point out, however, that there is evidence in the literature that a marked difference in the response of both adipose tissue and diaphragm to insulin may occur depending upon whether or not the medium employed is buffered by a bicarbonate-CO_2 mixture. For example, both Haugaard and Marsh (11) and Breibart and Engel (12) failed to find an increase in the R. Q. of rat retroperitoneal adipose tissue in a phosphate-buffered medium upon the addition of insulin. Beloff-Chain *et al.* (13) reported that the $C^{14}O_2$ production from uniformly C^{14}-labeled glucose by rat interscapular brown adipose tissue incubated in phosphate-buffered saline was not increased by insulin although fatty acid and glycogen synthesis were stimulated under these conditions. Winegrad and Renold (2) observed that insulin stimulated lactic acid production by rat epididymal adipose tissue only when it was incubated in a bicarbonate containing medium and not in a phosphate medium. This behavior of adipose tissue is of interest in relation to the reported requirement for bicarbonate by a highly purified fat synthesizing enzyme system from avian liver (Gibson *et al.* (14)). However, glucose

utilization and glycogen synthesis by rat diaphragm also appear to be sensitive to the presence or absence of bicarbonate in the medium. Beloff-Chain *et al.* (15) found the rate of glucose utilization and glycogen synthesis by this tissue to be greater in a bicarbonate medium than in a phosphate-buffered medium. The two glycolytic systems, the one insulin-sensitive and the other insulin-insensitive described by Shaw and Stadie (16) in rat diaphragm incubated in a phosphate buffered medium were not apparent when the diaphragm was incubated in a bicarbonate containing medium (17). It is thus apparent that further investigations are needed on the possible role of the CO_2-bicarbonate system on the response to insulin to be obtained *in vitro* with both adipose tissue and the diaphragm.

Adipose tissue would appear to be more sensitive to changes in the pH of the medium than diaphragm. Stadie and Zapp (6) reported that alterations in pH of phosphate-buffered saline solution from 6.3 to 7.6 did not alter the stimulation by insulin of glycogen synthesis in the rat diaphragm. However, a comparison of the two tissues in this respect is complicated by the use of a phosphate buffered medium in the case of the diaphragm and a bicarbonate medium in the case of the adipose tissue. There is a suggestion from our results that the response of adipose tissue to insulin is less affected by lowering the pH by changes in CO_2 tension than by lowering the bicarbonate content of the medium.

It may be concluded from the results presented here that the most suitable incubation medium of those tested for the stimulation of the epididymal fat pad by insulin *in vitro* is the bicarbonate-buffered Ringer solution of Krebs and Henseleit (4). It must be emphasized, however, that this conclusion applies only to the inorganic composition of the medium under conditions where insulin is added in excess of that required to produce a maximal stimulation. As shown in the preceding paper, it is very important when working with insulin concentrations within the physiological range to employ a medium containing gelatin or perhaps some other protein material.

The decreased response of the epididymal fat pad to insulin with increasing age of the rat observed here is an interesting and puzzling one. We have not investigated the response of rats weighing less than 150 gm. since the amount of tissue to be obtained from them is not usually sufficient to provide four pieces from a single rat. A change in weight from 150 to 250 gm. occurs in a span of about 3 weeks in the strain of rat that we have employed. The change in response of the tissue thus occurs within a rather short period of time. Whether dietary or hormonal factors are involved remains to be investigated. Ruska and Quast (18) have made observations on the R. Q. of rat epididymal adipose tissue obtained from rats ranging in weight from 100 to 400 gm. They found that the R. Q. progressively increased from values slightly below 1 to values in the neighborhood of 2 as the weight of the rat increased. We have not observed any appreciable variation in the R. Q. of epididymal adipose tissue of rats within the weight range 160 to 300 gm. as judged from its initial response before the addition of insulin. The mean initial response of the right distal portion of the epididymal fat pad in 58 rats has been -2.2 μl. per 100 mg. per hr. It should be noted, however, that the experiments of Ruska and Quast were carried out in a phosphate-buffered medium.

In the present experiments, where rats of similar weight and nutritional status were employed, no advantage was found in expressing the stimulation by insulin in terms of the total nitrogen content of the tissue rather than in terms of wet weight. This is probably a reflection of the fact that the nitrogen content of the epididymal fat pad in these rats appears to be fairly constant. In a total of 68 observations we have found a mean value of 2.63 (S.E. 0.122) mg. of nitrogen per gm. of wet tissue. However, it will undoubtedly be necessary to reinvestigate this point when comparisons are to be made on animals whose nutritional or hormonal status is other than normal.

SUMMARY

The effect of insulin upon the metabolism of glucose by epididymal adipose tissue of the rat was studied manometrically with particular reference to the influence of variations in the inorganic composition and pH of the medium. The results obtained indicate that a Krebs-Ringer bicarbonate medium is the most satisfactory of those tried. Minor variations in the composition of this medium produced insignificant changes in the tissue response to insulin. However, the tissue behaved abnormally and failed to respond to insulin in a medium containing potassium as the only cation. A comparison of the response to insulin to be obtained from four pieces of tissue from a single rat indicated that considerable variation may be encountered in certain instances, but in a series of experiments the mean values showed reasonable agreement. No advantage was to be found in the expression of activities in terms of total nitrogen rather than on the basis of tissue wet weight. Considerable variation in response is shown by adipose tissue from different rats and this is in part dependent upon the age of the rat.

REFERENCES

1. Ball, E. G., Martin, D. B., and Cooper, O., *J. Biol. Chem.*, **234**, 774 (1959).
2. Winegrad, A. I., and Renold, A. E., *J. Biol. Chem.*, **233**, 267 (1958).
3. Winegrad, A. I., and Renold, A. E., *J. Biol. Chem.*, **233**, 273 (1958).
4. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
5. Aldridge, W. N., *Biochem. J.*, **67**, 423 (1957).
6. Stadie, W. C., and Zapp, J. A., Jr., *J. Biol. Chem.*, **170**, 55 (1947).
7. Oyama, J., and Grant, R. L., *Federation Proc.*, **17**, 286 (1958).
8. Ball, E. G., Strittmatter, C. F., and Cooper, O., *Biol. Bull.*, **108**, 1 (1955).
9. Flink, E. B., Hastings, A. B., and Lowry, J. K., *Am. J. Physiol.*, **163**, 598 (1950).
10. Hastings, A. B., Teng, C.-T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, **194**, 69 (1952).
11. Haugaard, N., and Marsh, J. B., *J. Biol. Chem.*, **194**, 33 (1952).
12. Breibart, S., and Engel, F. L., *Endocrinology*, **55**, 70 (1954).
13. Beloff-Chain, A., Catanzaro, R., Chain, E. B., Masi, I., and Pocchiari, F., *Selected Scientific Papers from the Istituto Superiore di Sanita*, **1**, 345 (1956).
14. Gibson, D. M., Titchener, E. B., and Wakil, S. J., *J. Am. Chem. Soc.*, **80**, 2908 (1958).
15. Beloff-Chain, A., Chain, E. B., Catanzaro, R., and Longinotti, L., *Biochim. et Biophys. Acta*, **14**, 259 (1954).
16. Shaw, W. N., and Stadie, W. C., *J. Biol. Chem.*, **227**, 115 (1957).
17. Shaw, W. N., and Stadie, W. C., *Federation Proc.*, **17**, 310 (1958).
18. Ruska, H., and Quast, A., *Arch. exptl. Pathol. Pharmakol.*, **179**, 217 (1935).

A Method for Incorporating Cholesterol and Other Lipides into Serum Lipoproteins *in Vitro*

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It has been reported that small amounts of solid cholesterol dissolve on prolonged incubation with human serum (1). However, the nature of this process has so far been unknown. Lack of solubility of cholesterol in water has necessitated the use of suspensions stabilized by detergents for the purpose of intravenous administration in metabolic studies. This procedure cannot be regarded as satisfactory, since, as shown in the present study, the administered cholesterol, unlike the physiological form bound in lipoproteins, has been found to disappear rapidly from circulation. In view of the need for a suitable method of preparation of lipide substances for metabolic studies the present work was carried out. It has been found possible to incorporate various lipides into serum and serum lipoproteins by transfer from a solid phase. Since the preparations obtained by the method described apparently have properties of solutions, the latter term will be used here to describe their physical state. The phenomena observed have possibly a basic significance in the transport mechanism of lipide substances *in vivo*.

EXPERIMENTAL

4-C^{14}-cholesterol was purchased from the Nuclear-Chicago Corporation and kept refrigerated in a dilute benzene solution. H^3-labeled cholesterol was prepared by the Wilzbach method (2) and purified by recrystallization from alcohol with pure carrier cholesterol and subsequent passage through the dibromide according to Schwenk and Werthessen (3). Both the 4-C^{14}- and the tritium-labeled cholesterols were identified by their infrared spectra. They were shown to be free of more polar oxidation products by chromatography on alumina according to the method of Fredrickson (4). H^3-labeled corticosterone, H^3-desoxycorticosterone, 4-C^{14}-progesterone, and 4-C^{14}-testosterone were extensively purified until chromatographically homogeneous.[1] Serum lipoproteins were prepared by fractional centrifugation (5) and dialyzed against 0.9 per cent sodium chloride. Serum albumin was human serum Fraction V.

In order to facilitate solubilization the lipide compound was dispersed in a 1:100 proportion by weight on Celite 545 by mixing a pentane solution of the compound with the carrier and evaporating the solvent. The treated Celite was subsequently shaken under nitrogen at 37° with whole serum, or a serum fraction in a proportion of 50 mg. of Celite to 1 cc. of liquid. Unless otherwise stated, the incubation continued for 20 hours, and the suspension was subsequently filtered through a Swinny filter. A measured amount of the filtrate was extracted with a mixture of alcohol and acetone, 1:1, and the radioactivity determined in a Packard scintillation spectrometer. In the case of cholesterol or other nonpolar sterols only negligible amounts of label were found in solution in the absence of protein. In some instances a chemical analysis of the protein and lipide moieties of the lipoproteins was carried out. Cholesterol was determined by the Sperry and Webb method (6), with an error not exceeding 2 per cent.

[1] These four materials were kindly supplied by Dr. R. Peterson.

RESULTS

In order to ascertain that the labeled material incorporated into serum by the method described was cholesterol the following experiments were carried out. Both 4-C^{14}- and tritium-labeled cholesterol were dissolved in two serum samples, the lipides of which were subsequently extracted with alcohol-acetone (1:1); saponified and the nonsaponifiable fractions were extracted with heptane. The latter were chromatographed on paper according to Neher and Wettstein (7) alongside samples of the two starting materials. When the chromatogram of the C^{14}-containing samples was radioautographed, a single radioactive spot was obtained in each case. The R_F values for these spots were identical and coincided with the single colored spot developed by spraying with phosphomolybdic acid. It has been reported that this paper chromatographic system clearly separates cholesterol from several dihydroxy- and trihydroxysterols, which are likely to be formed as a result of oxidation of cholesterol (8). Radioactivity on the chromatogram of the tritium-containing samples was detected by cutting the paper into sections, eluting with ethanol, and counting in the Packard scintillation counter. The tritium-labeled material isolated from incubated serum also appeared to be identical with the starting material.

It was found that distribution of cholesterol on the surface of a carrier greatly increased the rate of its dissolution in serum. Various amounts of Celite were impregnated with a constant amount of labeled cholesterol and then incubated for 2 hours with rat serum. As shown in Fig. 1, the incorporation of radioactive cholesterol increased rapidly with the amount of Celite used. Centrifugal fractionation of human and rat sera treated by this method with labeled cholesterol and subsequent determination of radioactivity in each of the fractions revealed that the largest part of the dissolved cholesterol was associated with the various lipoprotein classes (Table I). Isolated serum lipoproteins were shown to take up cholesterol in a similar fashion.

The amounts of labeled cholesterol dissolved by human and rat serum, serum albumin, and human S_f 3-8 lipoproteins[2] were

[2] Lipoproteins S_f 3-8 were floated in the ultracentrifuge at solvent densities $1{,}109 < d < 1{,}063$.

followed as a function of the time of incubation (Fig. 2). Serum albumin exhibited complex-formation with only small amounts of cholesterol, the saturation point being reached rather rapidly. Human serum dissolved considerably more cholesterol and the amount of label incorporated continued to rise after 48 hours. Isolated serum S_f 3-8 lipoproteins gave a similar time curve. The

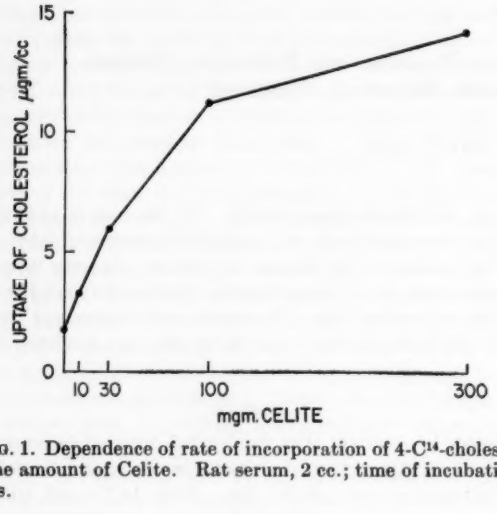

FIG. 1. Dependence of rate of incorporation of 4-C^{14}-cholesterol on the amount of Celite. Rat serum, 2 cc.; time of incubation, 2 hours.

TABLE I

Distribution of 4-C^{14}-cholesterol between centrifugal fractions in μg. per cc. of serum

Source	Fraction*	Cholesterol dissolved
Human serum	$d < 1.019$	40
	$1.019 < d < 1.063$	85
	$1.063 < d < 1.21$	17
	$d > 1.21$	5
Rat serum	$d < 1.21$	40
	$d > 1.21$	8

* Serum proteins were fractionated into the classes indicated, on the basis of density, d (5).

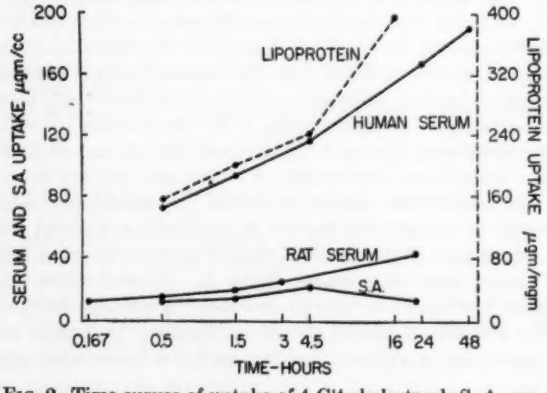

FIG. 2. Time curves of uptake of 4-C^{14}-cholesterol; S. A., serum albumin.

TABLE II

Uptake in vitro of sterols by albumin and lipoprotein S_f 3-8 in μg. per cc.

Compound	Solvents*		
	Control (buffer)	1 per cent albumin	1 per cent lipoprotein S_f 3-8
4-C^{14}-Δ^4-cholestenone	0	19	166
4-C^{14}-cholesterol	2	36	334
H^3-sitosterol	6	79	226
4-C^{14}-progesterone	16	150	363
4-C^{14}-testosterone	31	279	258
H^3-desoxycorticosterone	85	263	608
H^3-corticosterone†	149	642	730

* Serum albumin was dissolved in and lipoprotein dialyzed against phosphate buffer, pH 7.45, 0.16 M.

† Incubated with 100 mg. of impregnated Celite per cc. to exceed saturation of corticosterone.

TABLE III

Rise in content of nonesterified cholesterol in lipoproteins S_f 3-8 after incubation with cholesterol

	Free cholesterol in lipoproteins
	%
Control	9.0
Incubated	9.8
Control	10.2
Incubated	13.4

relatively large amount of cholesterol dissolved by 0.5 per cent lipoprotein solution again indicated that this fraction may be responsible for most of the solubilizing capacity of whole human serum.

Several other sterols and steroids were also taken up by serum lipoproteins, in most cases, to a greater extent than by an equal amount of serum albumin (Table II).

Isolated S_f 3-8 lipoproteins were incubated with 4-C^{14}-cholesterol-treated Celite, and the solution was analyzed for total lipoprotein and nonesterified cholesterol before and after the treatment (Table III). After the incubation procedure there was an increase in the percentage content of free cholesterol. This suggests that free cholesterol had been added to the pre-existing complex. It was shown by Hagerman and Gould (9) that serum-unesterified cholesterol, incorporated *in vivo*, exchanges with cholesterol of red cells after incubation *in vitro*. A similar experiment was carried out by incubating unlabeled rat red cells with rat serum containing 4-C^{14}-cholesterol incorporated *in vitro*. Samples were withdrawn at various times, and the specific activities of free cholesterol in serum and cells were determined. The results presented in Fig. 3 reveal a progressive exchange of labeled cholesterol similar to that reported for serum labeled *in vivo* (9).

In order to examine the usefulness of the method for metabolic studies the distribution of labeled cholesterol in rat tissues was studied after administration of the label in various forms. 4-C^{14}-cholesterol incorporated into serum *in vitro* by the present method was given intravenously simultaneously with a suspension of

tritium-labeled cholesterol, which was prepared by injecting rapidly an alcoholic solution of cholesterol into 0.9 per cent NaCl. Two minutes later the animal was killed, several of its organs homogenized in an alcohol-acetone mixture, and the H^3:C^{14} ratio in the total lipide extract of various tissues determined. In all the organs examined the uptake of the cholesterol suspension (H^3-labeled) was greater than that of the dissolved cholesterol (C^{14}-labeled) which was the main form remaining in circulation (Table IV).

In a second experiment three rats each received intravenously a different form of a preparation of labeled cholesterol. One animal was administered serum obtained from a rat previously fed H^3-labeled cholesterol; the second rat received serum in which 4-C^{14}-cholesterol was dissolved *in vitro* and the third one, a suspension of H^3-labeled cholesterol in 0.9 per cent NaCl. The animals were bled periodically and the concentrations of the isotopes in the lipide extracts of the serum samples were determined (Fig. 4). The suspended cholesterol almost completely disappeared from circulation, then gradually reappeared. On the other hand the shape of the disappearance curve of C^{14}-cholesterol dissolved *in vitro* resembled that of the cholesterol incorporated into lipoproteins *in vivo*. Allowance should be made, however, for the fact that the *in vitro* preparation contained the labeled compound exclusively in an unesterified form and therefore was subject during the initial phase of the experiment to more rapid exchange with tissue cholesterol than the natural preparation, in which most of the labeled cholesterol was esterified (10).

Serum labeled *in vitro* with dissolved cholesterol has been administered to several human subjects for metabolic studies and here also no excessive disappearance of the label from circulation was observed.[3]

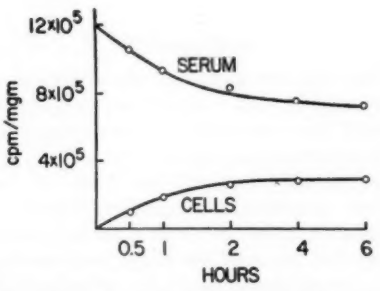

FIG. 3. Exchange of unesterified cholesterol incorporated *in vitro* into rat serum with cholesterol of red cells.

TABLE IV

*Soluble and suspended cholesterol in rat tissues**

Tissue	Ratio c.p.m. suspended (tritium): c.p.m. soluble (C^{14})
Serum	0.09
Kidney	4.41
Heart	5.44
Liver	5.83
Muscle	7.10
Spleen	8.13
Small intestine	8.88
Mixture injected	4.0

* See text for experimental conditions.

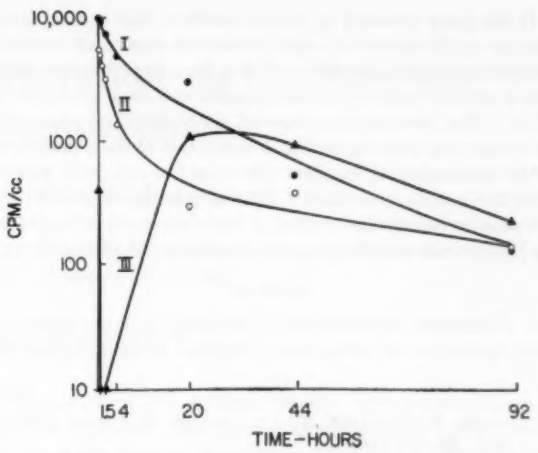

FIG. 4. Serum concentration of cholesterol administered in various forms to rats. Type of preparation and amounts of label injected:

I, serum obtained from a donor animal fed with 4-C^{14}-cholesterol (6×10^4 c.p.m.); *II*, serum labeled *in vitro* with 4-C^{14}-cholesterol (1.2×10^5 c.p.m.); *III*, a suspension of tritiated cholesterol (1×10^6 c.p.m.).

DISCUSSION

The results presented indicate that most of the capacity of serum for dissolving cholesterol under the conditions described can be attributed to the lipoproteins, particularly to the low density fractions. The fact that human serum is a better solvent than rat serum may be explained on the basis of the higher concentration of low density lipoproteins in man. Lack of specificity with respect to the type of lipide incorporated suggests that the phenomenon results from physical solution of the substance in the lipide phase of lipoproteins rather than from an interaction with specific sites on the protein moiety of the molecule.

The concentration of labeled cholesterol which could be incorporated into serum by this method *in vitro* is far larger than the concentration of label which might be achieved in serum by feeding or injecting comparable quantities of labeled cholesterol. Other sterols and steroids, some not normally present in the circulation and not absorbed by the organism, may also be dissolved *in vitro*.

Labeled cholesterol dissolved in serum *in vitro* resembles cholesterol incorporated *in vivo* with respect to two properties examined by us. (*a*) Neither form disappears rapidly from circulation when injected intravenously. Cholesterol in the form of a suspension, on the other hand, is rapidly removed by the tissues from serum. (*b*) The characteristic ability of unesterified serum cholesterol to exchange with cholesterol of red cells (9) is shared by labeled cholesterol incorporated into serum *in vitro*.

Circulating lipoproteins have ample opportunity to come in contact with lipides on the surface of cells. If an addition of cholesterol to lipoprotein complex takes place also *in vivo*, the phenomenon might partially contribute to the transport of cholesterol in the body.

[3] D. Steinberg and J. Avigan, unpublished results.

It has been reported by several authors that normal human sera on equilibration with solid cholesterol showed an increase in cholesterol content, whereas with sera from hypertensive individuals a drop in cholesterol concentration was often produced (*e.g.* (11)). The phenomenon observed with abnormal sera can be reasonably explained as partial denaturation of the β-lipoproteins in the sera examined, an effect that would be especially apparent when the latter are elevated. This drop is superimposed on the elevation in percentage content of free cholesterol in the remaining lipoproteins resulting from the treatment with the solid sterol.

SUMMARY

1. Cholesterol dispersed on Celite dissolves in the presence of whole human or rat serum and of isolated serum lipoproteins.

2. Most of the cholesterol dissolved by serum is associated with lipoprotein fractions.

3. Similarly to cholesterol, some other lipides can be solubilized in serum, or serum lipoproteins.

4. Cholesterol incorporated into serum lipoproteins by the present method behaves, when administered intravenously, more like cholesterol incorporated biosynthetically than does labeled cholesterol in the form of a suspension.

5. It is suggested that the procedure may serve as a convenient and useful method for labeling or modifying lipoproteins.

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REFERENCES

1. Loeper, M., Lemaire, A., and Lesure, A., *Compt. rend. soc. biol.*, **98**, 101 (1928).
2. Wilzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013 (1957).
3. Schwenk, E., and Werthessen, N. T., *Arch. Biochem. Biophys.*, **40**, 334 (1952).
4. Fredrickson, D. S., *J. Biol. Chem.*, **222**, 109 (1956).
5. Havel, R. J., Eder, H. A., and Bragdon, J. H., *J. Clin. Invest.*, **34**, 1345 (1955).
6. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, **187**, 97 (1950).
7. Neher, R., and Wettstein, A., *Helv. Chim. Acta*, **35**, 276 (1952).
8. Riddell, C., and Cook, R. P., *Biochem. J.*, **61**, 657 (1955).
9. Hagerman, J. S., and Gould, R. G., *Proc. Soc. Exptl. Biol. Med.*, **78**, 329 (1951).
10. Gould, R. G., *Am. J. Med.*, **11**, 209 (1951).
11. Eck, M., and Desbordes, J., *Compt. rend. soc. biol.*, **117**, 429 (1934).

The Effects of Thyroxin Administration on the Enzymic Reduction of Δ^4-3-Ketosteroids*

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The enzymic inactivation of Δ^4-3-ketosteroids involves three consecutive reactions: (*a*) saturation of the 4-5 double bond (2); (*b*) reduction of the carbonyl group at 3 to an alcohol (3); and (*c*) formation of a glucosiduronic acid with the "tetrahydrosteroid" (4). It has been reported that the double bond saturation is considerably slower than the carbonyl reduction and may therefore be the rate-limiting reaction in steroid degradation (2). Because of reports by Levin and Daughaday (5) and Peterson (6) that steroid turnover was accelerated in thyrotoxicosis, the effect of thyroxin administration on the enzymic reduction of the 4-5 double bond was investigated (7).

Evidence will be presented that thyroxin administration increases the rate of steroid reduction in two ways: (*a*) by causing an increase in available reduced triphosphopyridine nucleotide which is a rate-determining reactant in this process, and (*b*) by causing an increase in a steroid 5α-hydrogenase in liver microsomes.

EXPERIMENTAL

Materials—Androst-4-ene-3,17-dione was obtained from Nutritional Biochemicals Corporation. 5β-Dihydrocortisone (17α,21-dihydroxy-5β-pregnane-3,11,20-trione) and 5β-tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione) were supplied by Merck and Company. 5α-Dihydrocortisone (17α-21-dihydroxy-5α-pregnane-3,11,20-trione) was U.S.P. reference standard, No. 292. 3α-OH-5α-tetrahydrocortisone (3α,17α,21-trihydroxy-5α-pregnane-11,20-dione) was prepared by the enzymic reduction of 5α-dihydrocortisone (2). Other steroids were obtained from Sigma Chemical Company. All steroids employed were chromatographically homogenous. Sodium *l*-thyroxin, DPN, DPNH, TPN, and TPNH were obtained from the Sigma Chemical Company. *dl*-Isocitric acid was obtained from Nutritional Biochemicals Corporation. Isocitric dehydrogenase was prepared by extracting beef heart acetone powder with 10 volumes of 0.1 M sodium phosphate buffer, pH 7.

Identification of Products of Reaction—Steroids were subjected to paper chromatography according to modifications[1] of the Bush system (8). C_{21} steroids were separated in three systems: cyclohexane-benzene-methanol-water (two combinations); and benzene-methanol-water. They were detected by a spray of 0.5 per cent blue tetrazolium in 2.5 N ethanolic KOH. C_{19} steroids were separated in two solvent systems; cyclohexane-methanol-water, and cyclohexane-nitromethane-methanol. They were located by spraying the paper with Zimmerman's reagent (9). α,β-Unsaturated 3-ketosteroids were detected as quenching areas in ultraviolet light and by soda fluorescence (8).

Products of reactions were identified on the basis of identical mobility in more than one chromatographic system with reference steroids. In two instances, 3α-hydroxy derivatives were produced enzymatically for more satisfactory identification.

Animals—Male Sprague-Dawley rats that weighed 45 to 55 gm. at the beginning of the experiments received intraperitoneal injections of *l*-thyroxin, 0.5 μmole per day. They were fed Purina chow *ad libitum*. A 10^{-3} M solution of thyroxin in water was prepared at pH 10. When the solution was neutralized, a fine, flocculent precipitate resulted which was dispersed by shaking before the injections were given. In preliminary experiments, control rats received injections of sterile water. This was found to be unnecessary and in later experiments uninjected controls were used.

Rats that had received thyroxin failed to gain weight as rapidly as the controls. The ratios of liver to body weight were approximately the same in both groups.

Assay of Δ^4-3-Ketosteroid Hydrogenase—The animals were killed by decapitation. The livers were removed and homogenized in 2 volumes of 0.25 M sucrose at 0–5° in a glass homogenizer with a motor driver Teflon pestle.

Steroid reductase activity was determined by measuring in a Beckman model DU spectrophotometer the decrease in optical density at 240 mμ which occurred with the saturation of the 4-5 double bond as previously described (2). Enzyme, buffer, and steroid (which had first been dissolved in methanol and diluted with 9 volumes of water) were mixed and when TPNH was included, it was either added as such or generated by a system consisting of 1.5 μmoles of TPN, 5 μmoles of $MgCl_2$, 0.2 ml. of isocitric dehydrogenase, and 50 μmoles of isocitric acid.

While the reaction tube was still in ice, an aliquot was pipetted into 4 ml. of methylene chloride (purified by passage through silica gel) in a glass-stoppered centrifuge tube which was then shaken to extract the steroid from the aqueous phase. The tube containing the remainder of the mixture was incubated at 37°, usually for 5 minutes at which time a second aliquot was removed and extracted similarly. The extracted aliquots were centrifuged and the aqueous phase aspirated. The difference in optical density at 240 mμ between the initial and final aliquot indicated the amount of steroid reduced. Substrate reduction

* A preliminary report was presented at a meeting of the Federation of American Societies for Experimental Biology (1).

[1] R. E. Peterson, personal communication.

was constant for at least 10 minutes and was proportional to the amount of enzyme added.

Glucose-6-Dehydrogenase Assay—Glucose-6-P dehydrogenase was assayed in the supernatant fraction from liver homogenates centrifuged 100,000 × *g* for 1 hour (10).

Liver Slice Experiment—1.5 gm. of liver slices, approximately 0.05 mm. in thickness, were added to each of two 25-ml. beakers containing 0.5 mmole of potassium phosphate buffer, pH 7.4, 5 μmoles of $MgCl_2$, and 10 μmoles of glucose, in a total volume of 5 ml. To one beaker, 0.5 μmole of triiodothyronine was added. After 2 hours at 37° in a Dubnoff metabolic shaker, the slices were removed, homogenized, and assayed in the presence of excess TPNH for steroid reductase activity.

RESULTS

Early Effect—Liver homogenates from rats treated only 3 days with thyroxin reduced cortisone faster than homogenates from control rats. When excess TPNH was present however, the rates of cortisone reduction in these two preparations were identical (Table I, Columns 1 and 2). The effect of increasing TPNH concentration on the rate of cortisone reduction in the homogenates is seen in Fig. 1. Added TPNH greatly increases the rate of steroid reduction, indicating that its concentration in this system controls the rate of the reaction. It appeared from this experiment that an increase in TPNH available for this reduction could cause the increase in rate observed in the homogenate from the thyroxin-treated rat.

TABLE I

*Effect of thyroxin administration on rate of steroid reduction**

	Treated 3 days		Treated 20 days	
	Without added TPNH	With generated TPNH	Without added TPNH	With generated TPNH
Control	10	208	12	212
Thyroxin	35	215	72	452

* Steroid reductase was assayed after different intervals of thyroxin administration and expressed as mμmoles of cortisone reduced per minute per gm. of liver. Reaction mixtures in Columns 1 and 3 contained 20 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.4, 0.25 μmole of cortisone, 0.4 ml. of liver homogenate in a total volume of 1.1 ml. Reaction mixtures in Columns 2 and 4, contained 20 μmoles of tris(hydroxymethyl)-aminomethane buffer, pH 7.4, 0.2 ml. liver of homogenate, 0.3 μmole of cortisone, a TPNH-generating system, and 1.5 μmoles of TPN. Total volume 2 ml.

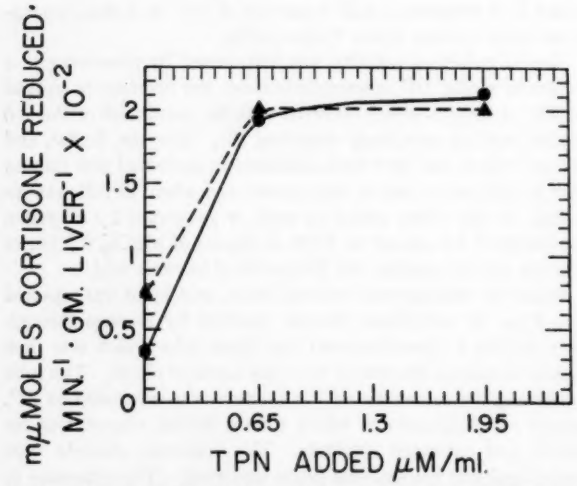

FIG. 1. Rate of steroid reduction as a function of TPNH concentration. Components identical to those in Table I, column 2, except for TPN. ●——●, uninjected control; ▲——▲, thyroxin treatment 3 days.

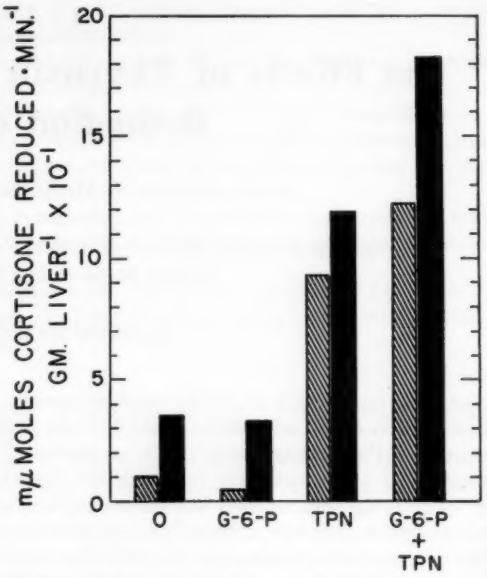

FIG. 2. Effect of glucose-6-P and TPN on the rate of cortisone reduction. Reaction mixture contained 20 μmoles of potassium phosphate buffer, pH 7.4; 0.3 μmole of cortisone, and 0.4 ml. of liver homogenate, total volume 1.4 ml. Where indicated, 25 μmoles of glucose-6-P and/or 0.5 μmole of TPN was added. The hatched bars represent normal liver homogenate; the solid bars, homogenate from rats treated 4 days with thyroxin.

Glucose-6-P dehydrogenase forms TPNH in the process of dehydrogenating glucose-6-P. For this reason, the specific activity of liver glucose-6-P dehydrogenase was measured and found to be doubled after 3 days of thyroxin administration.[2] Glock and McLean (11) had previously reported similar increases in this enzyme following 8 days of thyroxin treatment.

It was therefore of interest to determine whether this increase in glucose-6-P dehydrogenase could actually cause an increased rate of steroid reduction. In Fig. 2 the increased activity of the homogenate from the thyroxin-treated rat is indicated by the first pair of bars. Glucose-6-P did not affect the reaction in either homogenate but on addition of an excess of TPN, alone or with glucose-6-P, there was considerable stimulation of the rates of steroid reduction in both homogenates. The control, however, never attained the activity of the homogenate from the thyroxin-treated animal. It should be noted that in the presence of excess TPNH the steroid-reducing activity of the two homogenates is identical (Fig. 1). This demonstrated that in the presence of excess TPN and glucose-6-P, steroid reduction pro-

[2] Glucose-6-P dehydrogenase was also increased in adrenals of thyroxin-treated rats; unpublished observations.

ceeded faster in the homogenate containing increased glucose-6-P dehydrogenase.

Late Effect—When liver homogenates from rats treated with thyroxin for 20 days were assayed, an increased rate of steroid reduction was again observed (Table I, Columns 3 and 4). In this case however, with excess TPNH, the homogenate from the thyroxin-treated rat remained about twice as active as the control homogenate even though both were greatly stimulated, Fig. 3. Therefore, the faster rate observed in the homogenate from the thyroxin-treated rat could no longer be explained merely by increased available TPNH. This effect, although sometimes seen before 16 days, was consistently found at 20 days and could be produced by triiodothyronine administration for similar periods.

Liver homogenates, from rats that had received cortisone, 0.5 μmole daily for 17 days, when assayed for steroid reducing activity in the presence of excess TPNH were identical to control homogenates.

The possibility of an enzyme activator which might have been present in the homogenate from the thyroxin-treated rat was ruled out by the following experiment. Conditions were the same as described in Table I, Column 4. The activity of the control was 180, the thyroxin-treated, 420. When half-aliquots of each were mixed and assayed, the activity was 310, an insignificant variation from the predicted 300.

The intracellular location of the steroid reductase activity was determined in thyroxin-treated and normal rat livers (Table II). Half of the steroid reductase activity of the control homogenate was accounted for in the soluble fraction. The activity of this fraction from the treated rat, although identical to the control, represented only 24 per cent of the total activity of the liver, the majority being present in the particulate fraction.

All of the foregoing experiments were carried out using cortisone and androst-4-ene-3,17-dione as substrates. When tested, similar results were obtained with other Δ^4-3-ketosteroids (hydrocortisone, deoxycorticosterone, testosterone, and corticosterone).

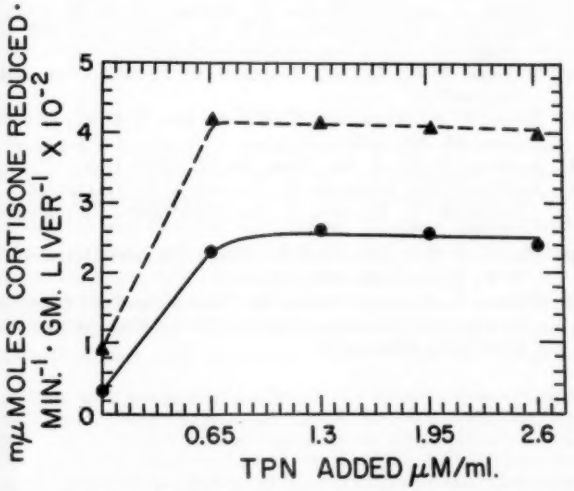

FIG. 3. Effect of TPNH concentration on the rate of steroid reduction in liver homogenates following 20 days of thyroxin treatment. ●——●, uninjected control; ▲——▲, thyroxin treatment. Reaction mixture identical to Table I, columns 3, 4, except for TPN.

TABLE II

Distribution of steroid reductase activity in liver*

	Control	Thyroxin treated for 20 days
Homogenate	225	455
Particulate fraction	98	299
Soluble fraction	115	110

* After centrifugation at 100,000 × *g* for 60 minutes, the particulate and supernatant fractions were assayed as described in "Methods" with excess TPNH. The activity is expressed as mμmoles of cortisone reduced per minute per gm. of liver. The microsomal fraction was found to contain virtually all of the activity of the particulate fraction following further fractionation by a modification of the method of Schneider and Hogeboom (12).

TABLE III

*Configuration of products of steroid reduction by rat liver fractions**

Liver fraction	Products from cortisone	Products from androst-4-ene-3,17-dione
Soluble, normal	3α-OH,5β-tetrahydrocortisone	3α-OH,5β-etiocholanolone
Soluble, thyroxin treated	3α-OH,5β-tetrahydrocortisone	3α-OH,5β-etiocholanolone
Microsomes, normal	3α-OH,5α-tetrahydrocortisone, 3β-OH, 5α-tetrahydrocortisone	3β-OH,5α-epiandrosterone
Microsomes,† thyroxin-treated	Same as normal microsomes plus 5α-dihydrocortisone	5α-androstane-3,17-dione, 3α-OH, 5α-androsterone

* Conditions for the reduction and preparation of the soluble and microsomal fractions are given in Table I and "Methods."

Steroids not previously designated in text are androsterone, (3α-hydroxy-5α-androstan-17-one); epiandrosterone, (3β-hydroxy-5α-androstan-17-one); etiocholanolone, (3α-hydroxy-5β-androstan-17-one); and 3β-OH,5α-tetrahydrocortisone, (3β,17α, 21-trihydroxy-5α-pregnane-11,20-dione).

† 5α-Androstane-3,17-dione cannot be separated by paper chromatography from 5β-androstane-3,17-dione. The former steroid was identified after elution and reduction with rat liver 3α-hydroxysteroid dehydrogenase (3). The resultant compound was identical with androsterone (5α) which can be easily distinguished from etiocholanolone (5β) which would have resulted from the further reduction of 5β-androstane-3,17-dione (3).

5α-Dihydrocortisone produced by reduction of cortisone with thyroxin-treated microsomes was further reduced with rat-liver 3α-hydroxysteroid dehydrogenase (3) and a steroid identical to 3α-OH,5α-tetrahydrocortisone was produced.

To rule out any direct effect of thyroxin or triiodothyronine on the steroid reductase, these thyroid hormones at a concentration of 1.5×10^{-3} M were preincubated separately with the microsomal fraction from rat liver. In the presence of excess TPNH, with androst-4-ene-3,17-dione as the substrate, no difference in activity was found between the microsomes preincubated with either thyroid hormone, and the control in which the hormone had been replaced by water.

In another attempt to effect an increase in steroid reductase activity *in vitro*, liver slices were incubated with triiodothyronine (see "Methods") as this compound is thought to have a more direct metabolic effect than thyroxin (13). No change in enzyme activity was observed following this treatment.

Steric Course of Reduction by Cell Fractions—The products of the reduction of a representative C_{19} and C_{21} steroid by TPNH with the soluble fraction and the microsomal fraction of liver are listed in Table III. In every case, the soluble enzyme gave rise to products with the 5β configuration. The soluble enzymes in rat liver which catalyze this conversion have been described (2, 3). All of the products of steroid reduction with microsomes had the 5α configuration. The enzyme in rat liver microsomes necessary for this conversion has been described previously (14). The tetrahydrosteroids produced by the microsomes from thyroxin-treated rats were chiefly 3α-OH, while the control microsomes produced mainly 3β-OH products.

DISCUSSION

The experiments presented above indicate that in liver homogenates from both thyroxin-injected and normal rats, the enzymes responsible for reduction of Δ⁴-3-ketosteroids are not saturated with TPNH. Consequently, the rate of steroid inactivation is controlled by the concentration of available TPNH. This is particularly interesting since the rate of corticoid synthesis in the adrenal is also regulated by TPNH concentration (15). That TPNH controls both processes suggests a homeostatic mechanism in which the amount of physiologically active steroid would remain constant in the event of concomitant variations in TPNH concentration in liver and adrenal.

The increase in steroid reduction occurring in rats treated only 3 days with thyroxin appears to be satisfactorily accounted for by an increase in available TPNH. A possible explanation for the greater concentration of TPNH in the livers of thyroxin-treated animals was the increased activity of glucose-6-P dehydrogenase. The mechanism of this increase in glucose-6-P dehydrogenase activity is presently unknown, as is the case with a large number of enzymes influenced by thyroxin (16).

It is conceivable that a number of other processes, *e.g.* fatty acid synthesis (17) and drug metabolism (18), which are dependent upon TPNH would be similarly affected following thyroxin administration.

After 2 to 3 weeks of thyroxin administration, in addition to the increase in TPNH, there occurs a large increase in a microsomal Δ⁴-steroid hydrogenase (5α) with no change in the soluble Δ⁴-steroid hydrogenase (5β). As a result of the change in relative enzyme levels, the ratio of 5β- to 5α-reduced steroids should be decreased. The urinary metabolites of testosterone from patients receiving triiodothyronine do in fact exhibit this alteration in the ratio of 5α to 5β isomers (19).

Since the 5β-reduced C_{19} steroids are biologically inert whereas many of their 5α epimers are androgenic (20), an interesting consequence of the thyroxin-induced increase in steroid hydrogenase (5α) is the production of a greater proportion of reduced steroids with biological activity.

The mechanism of thyroxin in effecting an increase in the microsomal enzyme, although unknown, is not accompanied by an increase in microsomal nitrogen or number.

SUMMARY

1. The rate and products of steroid reduction have been compared in normal and thyroxin-injected rats.

2. The rate of steroid reduction in liver homogenates from both groups was found to be controlled by reduced triphosphopyridine nucleotide concentration.

3. An increase in the rate of steroid reduction was observed in liver homogenates from animals treated 3 days, and was explained by an increase in available reduced triphosphopyridine nucleotide.

4. Treatment for 16 days resulted in a large increase in a Δ⁴-3-ketosteroid hydrogenase (5α) in the microsomal fraction of liver.

REFERENCES

1. McGuire, J. S., *Federation Proc.*, **17**, 272 (1958).
2. Tomkins, G. M., *J. Biol. Chem.*, **225**, 13 (1957).
3. Tomkins, G. M., *J. Biol. Chem.*, **218**, 437 (1956).
4. Isselbacher, K. J., and Axelrod, J., *J. Am. Chem. Soc.*, **77**, 1070 (1955).
5. Levin, M. E., and Daughaday, W. H., *J. Clin. Endocrinol. and Metabolism*, **15**, 1499 (1955).
6. Peterson, R. E., *J. Clin. Invest.*, **37**, 736 (1958).
7. McGuire, J. S., and Tomkins, G. M., *Nature*, **182**, 261 (1958).
8. Bush, I. E., *Biochem. J.*, **50**, 370 (1952).
9. Zimmerman, W., *Z. Physiol. Chem.*, **233**, 257 (1935).
10. Kornberg, A., and Horecker, B. L., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. I*, Academic Press, Inc., New York, 1955, p. 323.
11. Glock, G. E., and McLean, P., *Biochem. J.*, **61**, 390 (1955).
12. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950).
13. Gross, J., and Pitt-Rivers, R., *Biochem. J.*, **53**, 652 (1953).
14. Forchielli, E., and Dorfman, R. I., *J. Biol. Chem.*, **223**, 443 (1956).
15. Haynes, R. C., Jr., and Berthet, L., *J. Biol. Chem.*, **225**, 115 (1957).
16. Knox, W. E., Auerbach, V. H., and Lin, E. C. C., *Physiol. Revs.*, **36**, 164 (1956).
17. Langdon, R. G., *J. Am. Chem. Soc.*, **77**, 5190 (1955).
18. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., LaDu, B. N., Mitoma, C., and Udenfriend, S., *Science*, **121**, 603 (1955).
19. Bradlow, H. L., Hellman, L., Zumoff, B., and Gallagher, T. F., *Science*, **124**, 1206 (1956).
20. Fieser, L. F., and Fieser, M., *Natural products related to phenanthrene*, 3rd edition, Reinhold Publishing Corporation, New York, 1949, p. 375.

Electrophoretic Behavior of Serum Amylase*

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There have been several indications (1, 2) that the amylase found in serum is derived from sources other than the pancreas or salivary glands. For this reason we wished to determine whether any indication of fractionation of serum amylase could be obtained by paper electrophoresis. Although several accounts of the fractionation of amylase in serum are found in the literature, some involving precipitation and some involving electrophoresis, these data sometimes do not agree with one another and in other cases are rather limited. Cattaneo and Bassani (3), using acetone fractionation at low temperature, found that amylase in human serum always accompanied the albumin fraction, in horse serum, the globulin fraction. Baker and Pellegrino (4) using paper electrophoresis with human serum found amylase principally in the slow portion of the γ-globulin with only small amounts in the more mobile fractions. Data for four subjects were presented. In paper electrophoresis with mouse serum, Delcourt and Delcourt (5) found amylase principally (about 75 per cent) in the β_1- and β_2-globulin fractions. Kunkel and Eberhard,[1] in a single run using powdered plastic Geon in zone electrophoresis with human serum, found amylase mainly in the albumin fraction and a small amount with the α_1-globulin but none with the γ-globulin fraction.

In this study, serum fractions obtained by paper electrophoresis were analyzed by a micromodification of the amylase method of Van Loon *et al.* (6). Experiments in which serum was mixed with human saliva or pancreatic juice suggest the presence of some serum amylase different from salivary or pancreatic amylase.

EXPERIMENTAL

Amylase activities were determined with use of a micromodification of the amyloclastic method of Van Loon *et al.* (6) developed for this investigation. In this modification, quantities were reduced to one-tenth of the original amounts. Into 10-ml. volumetric flasks was pipetted 0.9 ml. of the starch-buffer substrate; the flasks were placed in a water bath at 37° for 15 minutes and 1 ml. of the solutions containing enzyme was added to the test flasks. For whole human serum, this was customarily a 1:100 dilution with 0.9 per cent NaCl. After 15 minutes incubation in the 37°-water bath, 6 ml. of water were added followed by 0.8 ml. of a 1:1 dilution with water of Van Loon's working iodine solution (6). The flasks were filled to the marks and mixed, and the optical densities of the blue starch-iodine mixtures were determined at 660 mμ in a Model 6A Coleman junior spectrophotometer with the use of 10 × 75-mm., round cuvettes. For comparative purposes, a further modification in which pH 7.6 Veronal buffer (5.80 gm. of barbituric acid, 3.00 gm. of sodium diethylbarbiturate, and 1.93 gm. of NaCl per l.) was substituted for pH 7.0 phosphate in some determinations.

The paper electrophoresis apparatus used in most of the determinations was a plastic horizontal model designed and constructed by one of the authors (J. P. L.). Strips of Whatman 3 mm. filter paper were equilibrated with Veronal buffer at pH 8.6. Serum, 20 μl., was applied to each of four strips in a spot near the apex and 16 cm. from the anode. A current of 0.44 ma. per cm. of paper width was applied for 12 hours. At the end of this time two strips were removed, dried at 130° for 5 minutes, and stained with bromphenol blue for 3 minutes. With the dyed strips used as a guide, the other two strips were cut so that the albumin, α_1-, α_2-, β-, and γ-globulin fractions were separated. The filter paper strips bearing the separate fractions were placed in 15-ml. centrifuge tubes and the amylase eluted with 10 ml. of cold (10°) 0.9 per cent NaCl. The tubes were stoppered, shaken gently for 10 minutes and centrifuged at 1500 r.p.m. for 5 minutes to pack the filter paper in the bottom of the tube. Amylase determinations were carried out on these eluates, as well as the original sera, with the use of the micro method with both the pH 7.0 phosphate buffer and pH 7.6 Veronal buffer.

In some experiments (running eight strips instead of four) the albumin fractions from two strips were eluted with 5 ml. of a 1:100 dilution of the corresponding whole serum with 0.9 per cent NaCl and with 0.9 per cent NaCl alone for two other strips. Amylase activities of these eluates were determined by the usual method with pH 7.0 phosphate buffer.

Six runs were made in which the electrophoretic distributions were determined both in a normal serum and the same serum plus dilute saliva or a few drops of pancreatic fluid.

Normal human serum of known amylase activity was mixed with an equal volume of saturated ammonium sulfate solution and allowed to stand at 2° for 2 to 3 hours. The precipitated proteins were separated by centrifugation and the supernatant fluid carefully removed. The supernatant was dialyzed at 2° for 18 hours in a Visking cellulose bag against 0.9 per cent NaCl to remove ammonium sulfate. The precipitated proteins were washed twice with a 1:1 mixture of 0.9 per cent NaCl and satu-

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[1] Personal communication.

rated ammonium sulfate, centrifuged, redissolved in 0.9 per cent NaCl and likewise dialyzed against 0.9 per cent NaCl. Amylase activities of both fractions were determined.

The normal sera used in this investigation were collected from healthy technicians and hospital patients with various disorders excluding those with pancreatic involvement. The abnormal sera came from patients suffering from acute pancreatitis as diagnosed by physical symptoms and serum amylase and lipase determinations.

RESULTS

The micromodification of the amylase method of Van Loon *et al.* was developed for the very small amounts of sera used in electrophoresis and their fractions. Amylase determinations on normal human sera with both the macro and micro methods gave results in which the mean ratio of micro:macro values was 0.96, the differences not statistically significant. Comparison of amylase activities at pH 7.6 and pH 7.0 gave a ratio of 1.05, the difference not significant. It was previously demonstrated (1) that for both rat and mouse serum and tissue amylases, the activities were essentially equal in pH 7.0 phosphate and pH 7.6 Veronal buffers. Determination of serum amylase activities at pH's 6.4, 6.8, 7.0, 7.2 (phosphate buffers), 7.4, 7.6, 7.8 and 8.2 (Veronal buffers) indicated a rather broad optimal region. Activities from pH 7.0 to 7.6 were essentially the same, dropping off to about 85 per cent of the maximum at pH's 6.4 and 8.2. Similar curves were obtained for the amylases from both the albumin and γ-globulin fractions.

In Table I the average distributions of serum amylase in 18 normal sera and 9 sera from subjects with pancreatitis are shown. As one would predict from the pH activity determinations, the small differences at pH 7.0 and 7.6 are not significant. In normal serum there is more amylase in the albumin fraction than any other single fraction, although there are appreciable amounts in the others. Essentially identical results were obtained in five comparative trials with a Spinco model R paper electrophoresis apparatus (Durrum type). When one adds up the amylase in all electrophoretic fractions of normal sera, the total exceeds that determined for the whole serum by almost 3-fold (311 compared to 106). If the discrepancies were due to errors in measurement of the relatively small amounts of amylase in the various fractions, then chance variations in some cases should make the total of the fractional amylase values smaller than those for the corresponding whole sera. This was never the case. Furthermore, one would expect that possible denaturation during electrophoresis and incomplete elution of amylase from the paper strips would tend to make the total of the fractions a little less than the original whole. In fact, this was just what was observed in the sera from subjects with pancreatitis in which the recovery of amylase activity in the electrophoretic fractions was about 85 per cent of the corresponding whole sera. When the electrophoretic albumin fractions from normal sera were eluted from duplicate strips with 0.9 per cent NaCl containing whole serum and just 0.9 per cent NaCl alone, the amylase activities of the eluates with dilute serum were much lower than the eluates without serum. In one outstanding case the amylase level of whole serum was 108, that of the albumin fraction recovered by elution with the serum-NaCl mixture was 204, and that of the same albumin fraction eluted with just NaCl was 1640. In other trials, the differences were not quite so pronounced but still showed the inhibiting effect of the whole serum. Whereas the average whole serum amylase activity was 85 and the electrophoretically separated albumin fraction eluted with only 0.9 per cent NaCl averaged 87, the same albumin fractions eluted with and measured in the presence of dilute whole serum had an average activity of only 20.

When 20 μl. of serum applied to filter paper was eluted with 0.9 per cent NaCl without electrophoresis having occurred, the recovery of amylase activity was about 90 per cent. Elution of an equal size strip of blank filter paper gave an eluate with no amylase activity. Serum dialyzed exhaustively against 0.9 per cent NaCl showed only a small decrease in amylase activity which could be accounted for quantitatively on the basis of a slight increase in volume during dialysis. When human serum was dialyzed against distilled water, at least 95 per cent of the amylase activity was lost. Adding back enough NaCl to make its concentration in the dialyzed serum 0.9 per cent completely restores the lost activity.

The pattern of amylase distribution in these pancreatitis sera is not the same as in the normal sera. The percentage of amylase in the albumin fraction is lower, and that in the γ-globulin fraction is markedly higher. The absolute amounts in both the albumin and γ-globulin fractions are higher than in the normal with the greater increase found in the γ-globulin fraction. The differences in percentages and absolute amounts in the other fractions are relatively small and not significant. When either saliva or pancreatic fluid is added to normal serum, the greatest amount of added amylase (Table II) is found in the γ-globulin fraction. The lowering of amylase of the albumin and α_1-globulin fractions which is seen in most of these trials may be due just to dilution of the serum by the added dilute saliva or pancreatic juice. At any rate, the small variations in the fractions other than the γ-globulin are not considered significant.

Since the point of application of serum was chosen arbitrarily as the spot from which the γ-globulin fraction moved neither forward nor backward, the possibility was considered that the

TABLE I

Distribution of serum amylase in electrophoretic fractions of human serum proteins

	Whole Serum	Albumin	α_1	α_2	β	γ	Total
	Amylase units per 100 ml. of normal serum						
pH 7.0	106	125	40	41	54	51	311
pH 7.6	116	112	29	42	53	75	311
	Per cent of total in all fractions						
pH 7.0		40	13	13	17	16	
pH 7.6		36	9	14	17	24	
	Amylase units per 100 ml. of pancrealitis serum						
pH 7.0	685	191	53	51	78	201	574
pH 7.6	660	187	55	59	40	219	560
	Per cent of total in all fractions						
pH 7.0		33	9	9	14	35	
pH 7.6		33	10	11	7	39	

amylase found associated with this fraction might be there only because of strong adsorption to the cellulose of the paper. When the point of application of serum or serum plus saliva was moved 11 cm. closer to the cathode, the γ-globulin fraction moved forward 3 cm. in 16 hours. In 12 such trials, very little or no amylase remained at the point of application but had moved with the γ-globulin fraction. After dilute serum was shaken with either powdered cellulose or filter paper strips, amylase determinations on the supernatant fluid showed that no adsorption of the enzyme from solution had occurred.

In contrast to the electrophoretic results, fractionation of human serum proteins with saturated ammonium sulfate and determination of the amylase activities of the fractions (Table III) showed that the major portion of the amylase activity was found in the globulin fraction. Apparently the amylase associated with the electrophoretic albumin fraction does not have the solubility properties of albumins. Salivary amylase added to serum was likewise precipitated by half saturation with ammonium sulfate.

TABLE II

*Distribution of amylase in normal serum compared to serum plus saliva and serum plus pancreatic fluid**

	Amylase units per 100 ml of serum or serum mixture				
	Albumin	α_1	α_2	β	γ
Saliva added					
1. −	145	63	44	57	58
+	63	84	40	17	2180
2. −	104	0	26	0	45
+	62	24	72	48	1640
3. −	167	160	19	0	44
+	112	31	25	38	660
4. −	280	19	71	0	52
+	235	25	86	49	690
Pancreatic fluid added					
5. −	167	160	19	0	49
+	261	130	62	44	313
6. −	280	19	71	0	52
+	237	12	119	49	1050

* In each case, the values represent those of a given serum coupled with those of that same serum to which either dilute saliva or pancreatic fluid had been added.

TABLE III

Ammonium sulfate fractionation of serum amylase

Amylase units per 100 ml. of serum			
Whole serum	"Albumin" or soluble fraction	"Globulin" or insoluble fraction	Total Recovered
56	15	27	42
60	11	52	63
60	4	40	44
87	7	49	56
Average 66	9	42	51
290*	15	185	200

* Serum, 5 ml., plus 0.4 ml. of 1:100 dilution of saliva. Original serum amylase activity was 66.

DISCUSSION

The partition or distribution of amylase in human serum as determined in this study is not that indicated by Baker and Pellegrino (4). Whereas they found amylase principally in the γ-globulin fraction, our results indicate that the largest amount of amylase is associated with the albumin fraction. Our results then agree partially with the preliminary finding of Kunkel and Eberhard.[1]

The relatively large amounts of amylase in both the albumin and γ-globulin fraction indicate that two electrophoretically different amylases may be present in serum. It is possible, although unlikely, that the amylase in the fractions of human serum other than the albumin fraction represents only that which is trailing (7) on the paper. If this were so, then serum amylase should actually be associated electrophoretically only with the albumin fraction. When a dilute solution of human salivary amylase or pancreatic fluid is added to serum, practically all the added amylase, after electrophoretic separation, is found in the γ-globulin fraction. Very little of the added amylase is found in the faster moving fractions. If salivary and pancreatic amylases were the same as the principal component of serum amylase, then a large increase in the amylase of the albumin fraction should have been observed. Since this was not the case, serum amylase or some part of it must be regarded as differing, at least electrophoretically, from salivary and pancreatic amylases. One must then conclude that at least a portion of normal serum amylase is formed in some site other than the salivary glands or the pancreas, a conclusion previously reached (1) from observations in pancreatectomized-salivarectomized rats.

Bernfeld *et al.* (8) have found that human salivary and pancreatic amylases are identical by all criteria applied.

Recent reports of Miller and Copeland (9, 10) indicated that a purified bovine serum trans-α-glucosylase with maltase and amylase activities has the same mobility as the α_1-globulin fraction. This enzyme has no chloride requirement. However, when human serum or fractions of serum were dialyzed against distilled water to remove diffusible ions, at least 95 per cent of the amylase activity was lost. Adding back the appropriate amount of sodium chloride completely restores the lost activity. Thus, none of the amylase activity of human serum or its fractions can be attributed to trans-α-glucosylase.

During the process of electrophoresis of normal serum it appears that an amylase inhibitor has been removed. The observations that whole serum can inhibit the amylase activity of a fraction of that serum which has been separated electrophoretically leads almost inevitably to this conclusion. Furthermore, in every case, the sum of the amylase activities of the electrophoretically separated fractions of normal serum was greater than the amylase activity of the whole serum. In sera from subjects with acute pancreatitis, however, the sum of the amylase in the fractions approximately equaled the activity of the whole serum, and the presence of the inhibitor could not be demonstrated. The activity of salivary amylase, rather than being inhibited by serum, is actually enhanced (11) when serum-saliva mixtures are assayed, apparently because of the protective effect of the serum proteins. The inhibitor cannot be removed from normal serum by dialysis and so is probably a protein which may well be destroyed by the proteolytic enzymes released into the blood during pancreatitis. It may well be that the large increase in serum amylase activity that follows an attack of

acute pancreatitis is due not only to an actual increase in amylase but also to the removal of the inhibitor.

SUMMARY

1. The amylase of normal human serum is associated chiefly with the electrophoretic albumin fraction of the serum, whereas added salivary and pancreatic amylases are associated chiefly with the γ-globulin fraction. Therefore, the amylase of the albumin fraction must be formed in some site other than the salivary glands and the pancreas. However, the pH-activity curves of the amylases from the albumin and γ-globulin fractions are similar and the solubility of serum amylase in ammonium sulfate solution is similar to salivary amylase.

2. An amylase inhibitor, probably a protein, is present in normal human serum but not in serum from subjects with pancreatitis.

3. A micromodification of Van Loon's amyloclastic amylase determination is described.

REFERENCES

1. McGeachin, R. L., Gleason, J. R., and Adams, M. R., *Arch. Biochem. Biophys.*, **75**, 403 (1958).
2. Roe, J. H., Jr., Smith, B. W., and Treadwell, C. R., *Proc. Soc. Exptl. Biol. Med.*, **87**, 79 (1954).
3. Cattaneo, C., and Bassani, B., *Boll. soc. ital. biol. sper.*, **13**, 424 (1938).
4. Baker, R. W. R., and Pellegrino, C., *Scand. J. Clin. and Lab. Invest.*, **6**, 94 (1954).
5. Delcourt, A., and Delcourt, R., *Compt. rend. soc. biol.*, **147**, 1104 (1953).
6. Van Loon, E. J., Likins, M. R., and Seger, A. J., *Am. J. Clin. Path.*, **22**, 1134 (1952).
7. Sommerfelt, S. C., *Scand. J. Clin. and Lab. Invest.*, **5**, 299 (1953).
8. Bernfeld, P., Duckert, F., and Fischer, E. H., *Helv. Chim. Acta*, **33**, 1064 (1950).
9. Miller, K. D., *J. Biol. Chem.*, **231**, 987 (1958).
10. Miller, K. D., and Copeland, W. H., *J. Biol. Chem.*, **231**, 997 (1958).
11. McGeachin, R. L., *Biochim. et Biophys. Acta* **19**, 391 (1956).

A Simplified Procedure for the Partial Purification of Acetylcholinesterase from Electric Tissue*

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The protein components of the acetylcholine system which is essential for the generation of bioelectric potentials (1) are of biological interest. Although the active site of acetylcholinesterase has been studied extensively, very little is known about the physical and chemical characteristics of the enzyme molecule. In order to investigate the properties of the enzyme, it was desirable to develop a standardized, reproducible method which would give higher yields than had been obtained by the purification procedure described by Rothenberg and Nachmansohn (2). As in previous studies, the material used throughout this work has been the electric tissue of *Electrophorus electricus* introduced by Nachmansohn and his colleagues 20 years ago. The esterase from this source has been demonstrated to have the same kinetic characteristics as the acetylcholinesterase of other conducting tissue (3).

EXPERIMENTAL

Methods—The enzyme activity was assayed by the colorimetric method described by Hestrin (4) which is based on the quantitative reaction of acetylcholine with hydroxylamine in alkaline solution. In addition, the preparations of the highest potencies were assayed by a continual automatic titration method developed by Wilson (5). As the most potent preparations lost activity on dilution, the initial dilution was made in a solution of 0.05 per cent gelatin and 0.05 N NaCl and was then diluted further.

The protein concentration was determined by the Lowry *et al.* (6) modification of the Folin method. The protein *N* of highly purified preparations was determined by a micro-Kjeldahl procedure. The value for total activity was expressed in moles of acetylcholine chloride cleaved per hour. The specific activity was defined as the mmoles of acetylcholine chloride hydrolyzed per mg. of protein per hour.

Purification Procedure—Preliminary studies were made on the stability and solubility of an enzyme preparation that had a specific activity of 5. The stability of the enzyme was determined at two ammonium sulfate concentrations, *i.e.* 8.5 per cent and 15 per cent; at pH 2, 4, 6, 8, and 10 and at two temperatures, 90 and 23°. At 90° almost all of the activity was destroyed rapidly. The activity of the enzyme kept for 2 hours at 23° as a function of pH is shown in Fig. 1. The results of the pH-stability study made it possible to choose suitable hydrogen ion concentrations for the fractionation procedures. A careful investigation was made of the optimal ammonium sulfate concentrations to fractionate the protein solutions at the different pH values. The procedures of the second and third ammonium sulfate fractionations described below were based on these results.

Preparation of Extract—The mucin (7) which interferes with fractionation procedures was removed as follows (2): Small pieces of tissue were covered with toluene and stored in the refrigerator. The toluene was changed every few days for a period of 6 weeks. Then the toluene was decanted and the tissue was pressed between layers of cheesecloth to remove excess moisture. Tissue, 3 kg., was suspended in 7.5 l. of cold 5 per cent ammonium sulfate and ground in Waring Blendors for 4 minutes. The homogenized tissue was allowed to stand in the cold for 1 hour and then was centrifuged in a refrigerated centrifuge for 15 minutes at 3,000 × *g*. The supernatant fluid was decanted and filtered through cheesecloth. The solid material was re-extracted with 3 l. of cold 5 per cent ammonium sulfate and the solid residue removed by centrifugation and discarded. The specific activity of the combined supernatant solutions was 0.5. The values obtained were dependent upon the starting material. The range of the results can be seen in Table I.

First Ammonium Sulfate Fractionation—The subsequent operations were performed in the cold. While the combined supernatant solutions were being mixed with a magnetic stirrer, solid ammonium sulfate was added slowly to a concentration of 15 per cent. The solution was distributed in three aspirator bottles and the precipitate formed was allowed to separate overnight. Because of its low density, as compared with that of the ammonium sulfate solution, the precipitate rose to the surface on standing. The enzyme remained in the liquid phase. The bottom layer was withdrawn, centrifuged at 3,000 × *g* and passed through four layers of cheesecloth. The mixture in the top layer was treated in a similar fashion, but if the separation was incomplete, the centrifugal force was increased.

To the combined filtrates solid ammonium sulfate was added slowly to a concentration of 31 per cent and the mixture was allowed to stand overnight. The precipitate formed was collected by centrifugation for 25 minutes at 3,000 × *g* and further concentrated by centrifugation for 15 minutes at 40,000 × *g*. The precipitate was dissolved in cold distilled water and the solution adjusted to pH 7 with 1 N NaOH.

The total volume was made up to approximately 900 ml. and a clear solution was obtained by centrifugation for 10 minutes at

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40,000 × *g*. The precipitate was discarded. The specific activity of the supernatant solution was 6.

Second Ammonium Sulfate Fractionation—Although the preliminary studies had shown that at pH 4 and at 23° about half of the enzyme activity was lost, loss in enzyme activity could be prevented by carrying out the fractionation at 2° and with the use of small volumes in order to keep the enzyme solution at pH 4 for less than half an hour. To a 100-ml. portion of the above solution was added a volume of 50 per cent ammonium sulfate solution which would raise the concentration to 7 per cent; 1 N H_2SO_4 was added to adjust the pH to 4.1 (±0.1). The precipitate was removed rapidly by centrifugation at 41,000 × *g* and was discarded. The supernatant solution was adjusted to pH 6.1 (±0.1) with 1 N NH_4OH and to a 25 per cent ammonium sulfate concentration by the addition of an estimated volume of a 50 per cent solution of the salt. The precipitate formed was collected after 1 hour by centrifugation at 54,000 × *g*. The supernatant fluid was discarded and the precipitate was dissolved in cold water and made up to 10 ml. The specific activity was 17.

Third Ammonium Sulfate Fractionation—Four of the above fractions were combined (40 ml. volume) and an estimated volume of cold water was added to reduce the protein concentration to 1 per cent. To the solution was added a volume of a 50 per cent ammonium sulfate solution to raise the concentration to 14 per cent and 0.4 N H_2SO_4 was added to adjust the pH to 4.2. The precipitate formed was removed rapidly by centrifugation at 41,000 × *g* and was discarded. To the supernatant fluid was added 0.3 N NH_4OH to adjust the pH to 7.9 (±0.1) and a volume of 50 per cent ammonium sulfate solution to raise the concentration to 20.7 per cent. The precipitate formed was collected after 1 hour by centrifugation at 54,000 × *g*. The supernatant solution was discarded, the precipitate was dissolved in cold water, and the volume was brought to 5 ml. A few drops of toluene were added. The specific activity was 110.

The yields obtained are summarized in Table I.

The enzyme solution prepared by the third fractionation retains its activity for a period of several months if the protein concentration is diluted to 0.3 per cent and the enzyme is kept in a solution of 5 per cent ammonium sulfate and 0.01 M sodium phosphate buffer at pH 7.0. If it was desirable to store the enzyme for longer periods of time it was lyophilized. To this end the enzyme solution from the third fractionation was mixed with an equal volume of 0.09 M NaCl and 0.04 M Veronal buffer at pH 7.4. It was then dialyzed three times against 3 l. of the above buffer and then three times against 3 l. of 0.06 M NaCl and 0.025 M Veronal buffer at pH 7.3. After lyophilization the powder was stored in a desiccator at 4°. To redissolve the powder, 200 mg. of lyophilized protein were suspended in 10 ml. of 0.02 M sodium phosphate buffer at pH 7.0, allowed to stand for one-half hour, and dialyzed twice against 3 l. of the same buffer.

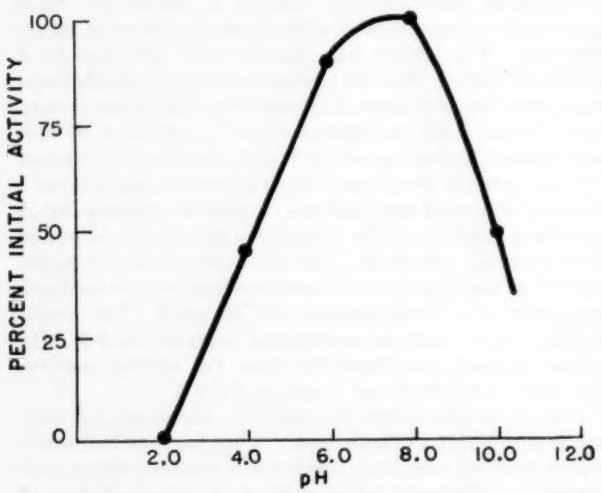

FIG. 1. The stability of a fairly crude preparation of acetylcholinesterase in 8.5 per cent ammonium sulfate, at 23°, is shown as a function of pH.

DISCUSSION

As a result of these studies, a standardized procedure has been developed which has made possible the preparation of highly purified material in a good yield. The limited quantity of source material available makes the yield very important. The most highly purified preparations reported in the earlier studies (2) and obtained by ultracentrifugation had a specific activity of 400. The preparations obtained by the described procedure have an average specific activity of 110; some preparations reached a value of 170. The material thus prepared is of adequate purity for many types of investigations, such as kinetic studies. Furthermore, the method developed has made it possible to obtain relatively large quantities of material suitable for isolation and characterization of the enzyme molecule.

TABLE I

Fractionation procedure

The values reported were obtained from a representative preparation of 3 kg. of toluene-treated eel tissue.

Fraction	Total activity recovered	Specific activity	Yields	
			Activity	Protein
	moles/hr.	*mmoles/mg./hr.*	%	%
Extract	74	0.5 (0.4–1.6)*	100	100
First ammonium sulfate fraction	41	6.0 (5–8)*	55 (50–70)*	4.6 (3.6–6.5)*
Second ammonium sulfate fraction	25	17.0 (14–18)*	34 (24–40)*	1.0 (0.6–1.2)*
Third ammonium sulfate fraction	15	110.0 (55–170)*	20 (10–24)*	0.09 (0.05–0.15)*

* Range of values obtained from a series of preparations.

SUMMARY

Acetylcholinesterase was extracted from the electric organ of *Electrophorus electricus* and subjected to ammonium sulfate fractionation yielding approximately 60 per cent of the initial activity with a specific activity of 6.0. A second ammonium sulfate fractionation at pH 4 and pH 6 increased the specific activity to 17. The third step increased the specific activity to 110 by ammonium sulfate fractionation at pH 4 and pH 7.9. A greater than 200-fold purification has been obtained with a 20 per cent yield.

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REFERENCES

1. Nachmansohn, D., *The Harvey lectures, Series 49* (1953–1954), Academic Press, Inc., New York, 1955, p. 57.
2. Rothenberg, M. A., and Nachmansohn, D., *J. Biol. Chem.*, **168**, 223 (1947).
3. Nachmansohn, D., and Rothenberg, M. A., *J. Biol. Chem.*, **158**, 653 (1945).
4. Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949).
5. Wilson, I. B., and Cabib, E., *J. Am. Chem. Soc.*, **76**, 5154 (1954).
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
7. Bailey, K., *Biochem. J.*, **33**, 255 (1939).

Preparation and Microbiological Properties of Tripeptides of β-2-Thienylalanine*

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Utilization of dipeptides of phenylalanine has been demonstrated in the growth of a *phenylalanineless* strain of *Escherichia coli* (1), in reversal of the toxicity of β-2-thienylalanine and its dipeptides in the growth of microorganisms (2–4), and in the synthesis of an adaptive enzyme (5). Dipeptides of β-2-thienylalanine competitively inhibited the utilization of dipeptides of phenylalanine (4, 5), and such inhibition studies have contributed additional evidence to previous proposals of a special role of peptides in the growth of microorganisms (6, 7) and indicate the possibility of specific sites of utilization of dipeptides.

Growth studies with *Lactobacillus arabinosus* (8) showed that L-leucyl-L-phenylalanylglycine was more effective than phenylalanine or the related dipeptides in reversing the toxicity of L-leucyl-β-2-thienyl-L-alanylglycine, and this was taken as evidence for possible utilization of tripeptides without previous hydrolysis to the free amino acids or the intermediate dipeptides. In order to pursue studies of the role of tripeptides in metabolism further we have now prepared four tripeptide analogues containing β-2-thienylalanine and tested their utilization in the growth of *E. coli* strain 9723.

Glycylglycyl-β-2-thienyl-DL-alanine, glycyl-β-2-thienyl-DL-alanylglycine, β-2-thienyl-DL-alanylglycylglycine, L-leucyl-β-2-thienyl-L-alanine, L-leucyl-β-2-thienyl-DL-alanine, L-leucyl-β-2-thienyl-L-alanylglycine, L-leucyl-L-phenylalanylglycine, and various intermediates leading to the peptides were prepared. The papain method of resolution (9–12) was found convenient for preparation of β-2-thienyl-L-alanine in amounts needed for the synthesis of peptides. Glycylglycyl-DL-phenylalanine, glycyl-DL-phenylalanylglycine (13) and DL-phenylalanylglycylglycine (14) were prepared by standard procedures.

EXPERIMENTAL

Preparation of Compounds

Table I summarizes the analytical data on the new compounds, which were prepared by standard procedures. The resolution of β-2-thienylalanine by the papain method offers the advantage of adaptability to large batches; the starting compound, *N*-acetyl-β-2-thienyl-DL-alanine, was conveniently prepared by basic hydrolysis of diethyl 2-thenylacetamidomalonate (15).

Coupling Procedure—The carbobenzoxy derivative of the amino acid or peptide was coupled with the amino acid ester in the presence of isobutyl chloroformate as directed by Vaughan and others (16, 17). The carbobenzoxy peptides were purified from ethyl acetate-petroleum ether.

Saponification of Esters—The ester was stirred at room temperature with 1 equivalent of 1 M NaOH and enough acetone to effect solution. When saponification appeared to be complete, the acetone was blown off, the aqueous solution was extracted with ether, acidified with HCl, and the carbobenzoxy peptide extracted into ethyl acetate and isolated.

Removal of Carbobenzoxy Group—A mixture of the carbobenzoxy peptide in warm nitromethane was saturated with anhydrous HBr (17). The resulting peptide hydrobromide was washed with ether, dissolved in ethanol or water, and neutralized with concentrated NH_4OH. The peptides containing leucine usually precipitated more readily from water than from ethanol. Peptides which did not contain sulfur were obtained by hydrogenolysis of the carbobenzoxy derivative in the presence of palladium black.

Resolution of β-2-Thienylalanine

1. N-acetyl-β-2-thienyl-L-alanine p-toluidide—63.9 gm. of *N*-acetyl-β-2-thienyl-DL-alanine were dissolved in 300 ml. of 1 M NaOH. The pH was adjusted to 4.5 with glacial acetic acid, and the mixture was combined at 40° with a solution of 43.2 gm. of *p*-toluidine hydrochloride in 250 ml. of 1 M acetate buffer (pH 4.5). To this clear solution was added 150 ml. of papain extract,[1] and the volume was increased to 1500 ml. with the acetate buffer. After incubation at 40° for 3 days, the precipitate was filtered off and washed successively with cold water, 1 M NaOH, and cold water. Yield, 40 gm.; m.p., 213° after purification from hot ethanol.

2. β-2-Thienyl-L-alanine—32.8 gm. of the above compound were refluxed for 4 hours with 6 N HCl. The acid was removed by repeated evaporation to dryness under reduced pressure on a water bath. The residue was dissolved in 100 ml. of water, made ammoniacal, and extracted with ether to remove the *p*-toluidine. After the solution was decolorized it was concentrated to a low volume and chilled overnight. The precipitate of β-2-thienyl-L-alanine was filtered off and washed with cold water, ethanol, and ether. Yield, 15.3 gm.; m.p., 266–67° (decomposition point); $[\alpha]_D^{28}$ −31.5° (25 mg. in 2.5 ml. of water). Literature values for rotation are −31.4 (18) and −31.7 (19, 20).

3. N-acetyl-β-2-thienyl-D-alanine—The filtrate from removal of the *N*-acetyl-β-2-thienyl-L-alanine *p*-toluidide was allowed to

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[1] 15 gm. of papain (Wallerstein) were stirred in an ice bath with 150 ml. of acetate buffer (pH 4.5) for 2 hours. Near the end of this time 7 gm. of L-cysteine hydrochloride were added. The clear filtrate was used.

TABLE I

Analytical data on compounds

Compound*	Formula	Yield	$[\alpha]_D^{\dagger}$	Calculated			Found			m.p.
				C	H	N	C	H	N	
		%		%	%	%	%	%	%	°C.
Cbz-glycylglycyl-β-2-thienyl-DL-alanine methyl ester	$C_{20}H_{23}O_6N_3S$	60		55.41	5.34	9.69	55.40	5.28	9.86	74
Cbz-glycylglycyl-β-2-thienyl-DL-alanine	$C_{19}H_{21}O_6N_3S$	75		54.40	5.05	10.02	54.20	5.05	10.11	157–158
Chloroacetylglycyl-β-2-thienyl-DL-alanine	$C_{11}H_{13}O_4N_2SCl$	72		43.35	4.30	9.19	43.67	4.36	9.16	148
Glycylglycyl-β-2-thienyl-DL-alanine	$C_{11}H_{15}O_4N_3S$	77		46.30	5.30	14.73	46.21	5.38	14.53	265–266‡
Cbz-glycyl-β-2-thienyl-DL-alanylglycine ethyl ester	$C_{21}H_{25}O_6N_3S$	72		56.36	5.63	9.39	56.60	5.79	9.46	98
Cbz-glycyl-β-2-thienyl-DL-alanylglycine	$C_{19}H_{21}O_6N_3S$	88		54.40	5.05	10.02	54.49	5.02	9.91	147
Glycyl-β-2-thienyl-DL-alanylglycine	$C_{11}H_{15}O_4N_3S$	77		46.30	5.30	14.73	46.16	5.23	14.47	233‡
Cbz-β-2-thienyl-DL-alanylglycylglycine ethyl ester	$C_{21}H_{25}O_6N_3S$	80		56.36	5.63	9.39	56.42	5.67	9.20	96
Cbz-β-2-thienyl-DL-alanylglycylglycine	$C_{19}H_{21}O_6N_3S$	31		54.40	5.05	10.02	54.59	5.19	9.82	142
β-2-Thienyl-DL-alanylglycyl-glycine	$C_{11}H_{15}O_4N_3S$	28		46.30	5.30	14.73	45.82	5.30	14.49	234‡
N-Acetyl-β-2-thienyl-L-alanine *p*-toluidide	$C_{14}H_{18}O_2N_2S$	89	+29.7	63.55	6.00	9.27	63.77	5.98	9.14	213
N-Acetyl-β-2-thienyl-D-alanine	$C_9H_{11}O_3NS$	78	−43.15	50.69	5.20	6.57	50.71	5.19	6.45	169
β-2-Thienyl-L-alanine methyl ester hydrochloride	$C_8H_{11}O_2NS\cdot HCl$	86	+38.1[a]	43.34	5.46	6.32	43.38	5.44	6.48	174
Cbz-L-leucyl-β-2-thienyl-L-alanine methyl ester	$C_{22}H_{28}O_6N_2S$	70	−10.2	61.08	6.52	6.48	60.92	6.45	6.51	108
Cbz-L-leucyl-β-2-thienyl-L-alanine	$C_{21}H_{26}O_6N_2S$	89	+3.4	60.27	6.26	6.69	60.22	6.26	6.60	132
L-Leucyl-β-2-thienyl-L-alanine	$C_{13}H_{20}O_3N_2S$	53	+32.8[b]	54.90	7.09	9.85	54.73	7.00	9.75	251‡
Cbz-L-leucyl-β-2-thienyl-L-alanylglycine ethyl ester	$C_{25}H_{32}O_6N_3S$	75	−27.3[c]	59.62	6.61	8.34	59.61	6.30	8.21	144
Cbz-L-leucyl-β-2-thienyl-L-alanylglycine	$C_{23}H_{29}O_6N_3S$	95	−42.1	58.09	6.15	8.84	57.91	6.24	8.89	173
L-Leucyl-β-2-thienyl-L-alanylglycine	$C_{15}H_{23}O_4N_3S\cdot H_2O$	34	+22.0[d]			11.66			11.43	215‡
Cbz-L-leucyl-L-phenylalanylglycine ethyl ester	$C_{27}H_{33}O_6N_3$	82	−24.4[e]			8.45			8.32	125
Cbz-L-leucyl-L-phenylalanylglycine	$C_{25}H_{31}O_6N_3$	96	−27.9			8.95			8.94	167
L-Leucyl-L-phenylalanylglycine	$C_{17}H_{25}O_4N_3$	100				12.53			12.75	203‡

* Cbz = carbobenzoxy.

† Optical rotations were determined at 26°, and the solutions were 1 per cent in absolute ethanol, except as noted: [a]At 32°; [b]20 mg. in 2 ml. of 1 per cent $NaHCO_3$; [c]at 29°; [d]1 per cent in water.

‡ Decomposition point.

stand for 3 days, and the small amount of precipitate which formed was discarded. The solution was heated to coagulate the enzyme, which was filtered off. The filtrate was made ammoniacal, extracted with ether, and concentrated under a stream of air to about one-half volume. Upon acidification, 24.5 gm. of *N*-acetyl-β-2-thienyl-D-alanine precipitated; m.p., 169° after purification from hot water.

4. β-2-Thienyl-D-alanine—22 gm. of the preceding compound were refluxed with 250 ml. of 6 N HCl for 4 hours. The acid was removed by repeated evaporation to dryness under reduced pressure on a water bath. The residue was taken up in ethanol and neutralized with concentrated NH_4OH to pH 6.8. Yield, 14.2 gm.; m.p., 265–266° (decomposition point); $[\alpha]_D^{28}$ +31.4° (25 mg. in 2.5 ml. of water). Literature values for rotation are +31.4 (18) and +31.6 (20).

E. coli Studies

The medium and procedure were identical with those employed previously in growth studies with *E. coli* strain 9723 (4). The test compounds were checked for homogeneity by paper chromatography and no contaminating amino acids or peptides were detected. All test compounds were added aseptically to previously sterilized salts-glucose medium, and the inoculated tubes were incubated at 37°. At the end of the growth period (16

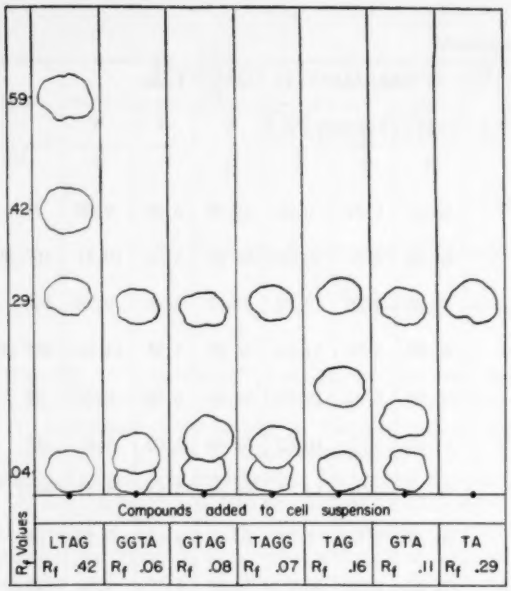

FIG. 1. Drawing of typical chromatograms showing the hydrolysis of peptides of β-2-thienylalanine in the presence of resting cells of *Escherichia coli* strain 9723. LTAG, L-leucyl-β-2-thienyl-L-alanylglycine; GGTA, glycylglycyl-β-2-thienyl-DL-alanine; GTAG, glycyl-β-2-thienyl-DL-alanylglycine; TAGG, β-2-thienyl-DL-alanylglycylglycine; TAG, β-2-thienyl-DL-alanylglycine; GTA, glycyl-β-2-thienyl-DL-alanine; and TA, β-2-thienyl-DL-alanine.

hours unless otherwise noted) the turbidity was determined with an instrument (21) in which distilled water reads 0 and an opaque object reads 100. The peptides were added in an amount so that 1 unit contained the equivalent of 1 μg. of β-2-thienyl-DL-alanine or DL-phenylalanine.

The procedure followed to determine the ability of *E. coli* strain 9723 to hydrolyze tripeptides of β-2-thienylalanine was similar to that reported for other peptides (22). A 24-hour culture of cells was washed with 0.9 per cent saline solution followed by phosphate buffer (pH 7) and then suspended in the buffer to give a cell concentration of 4.5 mg. per ml. 1 ml. of cell suspension was added to 1 ml. of peptide (1 mg. per ml.) and incubated at 37°. At intervals of 0, 60, 120, and 240 minutes 30-μl. samples of the incubating preparation were withdrawn and placed on Whatman filter paper for preparation of the chromatograms. *n*-Butanol saturated with water was used for the solvent, and the papers were developed with ninhydrin. The results are illustrated in Fig. 1. The R_F values were 0.04 for glycine and 0.59 for L-leucyl-β-2-thienyl-L-alanine; other R_F values are indicated in the figure. Tests with the dipeptides glycyl-β-2-thienyl-DL-alanine and β-2-thienyl-DL-alanylglycine were included for reference. After 1 hour, a faint but distinct spot of β-2-thienylalanine appeared from each of the peptides and became more pronounced with the 2- and 4-hour chromatograms. The only dipeptide detected as an intermediate in the hydrolysis of the tripeptides was L-leucyl-β-2-thienyl-L-alanine. The other dipeptides which were possible intermediates, glycyl-β-2-thienylalanine and β-2-thienylalanylglycine, did not appear on any of the tripeptide chromatograms. Free β-2-thienylalanine and glycine were readily formed under these conditions from each of the tripeptides and dipeptides added to the resting cells. At the end of 4 hours, distinct spots were still evident for each of the starting peptides, showing that complete hydrolysis had not occurred.

RESULTS AND DISCUSSION

The tripeptides of phenylalanine were more effective than free phenylalanine in reversing the toxicity of β-2-thienylalanine for *E. coli* (Fig. 2) and in this way resembled the behavior of dipeptides (3, 4). Whereas 30 μg. of DL-phenylalanine caused no reversal of the toxicity of β-2-thienyl-DL-alanine (1000 μg. per 5 ml.), an equivalent amount of each phenylalanine tripeptide produced 50 per cent growth or better.

The growth-stimulating effect of tripeptides does not necessarily result from previous conversion to the corresponding dipeptide or free amino acids, as is shown in Fig. 3. In this test the salts-glucose medium was supplemented with 3000 μg. of β-2-thienyl-DL-alanine and 3000 units of glycyl-β-2-thienyl-DL-alanine per 5 ml. Under these conditions, glycylglycyl-DL-phenylalanine was more effective than DL-phenylalanine, glycyl-DL-phenylalanine, or an equimolar mixture of DL-phenylalanine and glycyl-DL-phenylalanine in stimulating growth of *E. coli*.

When tested in the salts-glucose medium with no other supplements, glycylglycyl-β-2-thienyl-DL-alanine and glycyl-β-2-thienyl-DL-alanylglycine produced inhibition curves identical with that of β-2-thienyl-DL-alanine (Fig. 4); under these conditions all three compounds produced complete inhibition at a concentration of 3 units per 5 ml. β-2-Thienyl-DL-alanylglycylglycine and L-leucyl-β-2-thienyl-L-alanylglycine inhibited growth when added at 10 and 20 units per 5 ml., respectively. Although none of the tripeptides was more toxic than the parent amino acid when tested in the basal medium, all were much more toxic than free β-2-thienyl-DL-alanine or the corresponding dipeptides

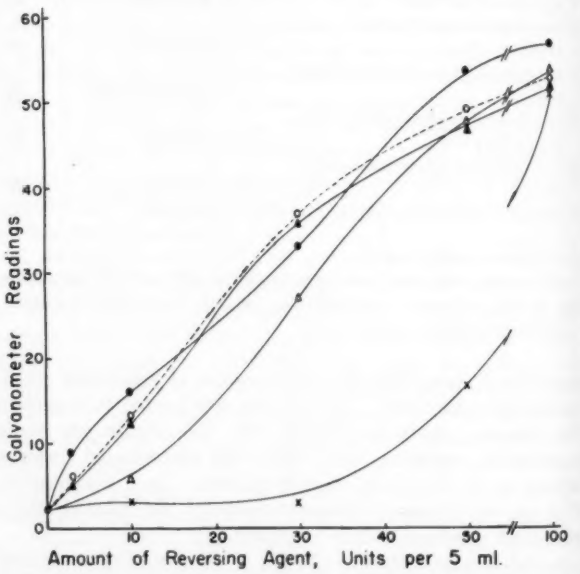

FIG. 2. Reversal of growth inhibition of β-2-thienyl-DL-alanine (1000 μg. per 5 ml.) in *Escherichia coli* strain 9723 with DL-phenylalanine (×), glycylglycyl-DL-phenylalanine (○), DL-phenylalanylglycylglycine (●), glycyl-DL-phenylalanylglycine (▲), and L-leucyl-L-phenylalanylglycine (△).

when tested in the presence of analogous tripeptides of phenylalanine.

Fig. 5 shows the toxicity of some of the tripeptides and related dipeptides of β-2-thienyl-DL-alanine when tested in the presence of glycylglycyl-DL-phenylalanine (30 units per 5 ml.). Under these conditions, glycylglycyl-β-2-thienyl-DL-alanine and glycyl-

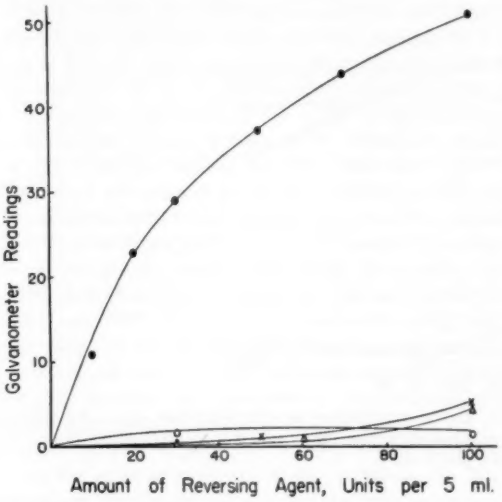

FIG. 3. Comparative growth-stimulating effect of glycylglycyl-DL-phenylalanine (●), glycyl-DL-phenylalanine (○), DL-phenylalanine (×), and an equimolar mixture of glycyl-DL-phenylalanine and DL-phenylalanine (△). The medium contained 3000 μg. of β-2-thienyl-DL-alanine and 3000 units of glycyl-β-2-thienyl-DL-alanine per 5 ml.

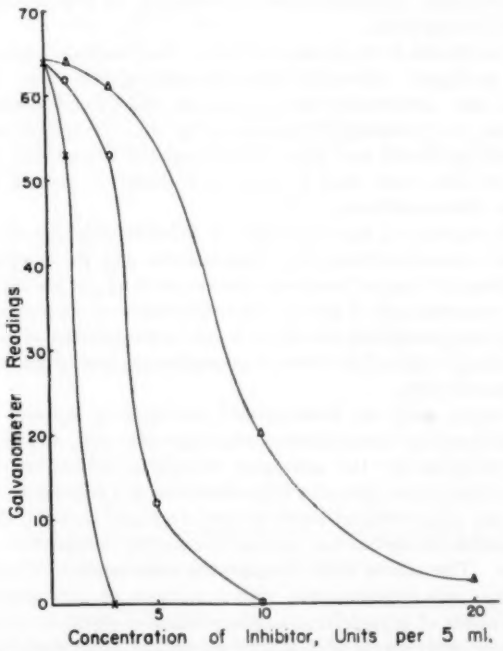

FIG. 4. Inhibition of growth of *Escherichia coli* with β,2-thienyl-DL-alanine (×), glycyl-β-2-thienyl-DL-alanylglycine (×)-glycylglycyl-β-2-thienyl-DL-alanine (×), β-2-thienyl-DL-alanylglycylglycine (○), and L-leucyl-β-2-thienyl-L-alanylglycine (△).

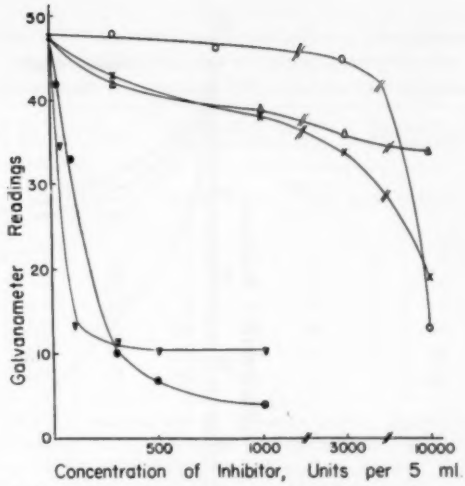

FIG. 5. Relative toxicity of β-2-thienyl-DL-alanine (×), β-2-thienyl-DL-alanylglycylglycine (○), glycyl-β-2-thienyl-DL-alanine (△), glycyl-β-2-thienyl-DL-alanylglycine (▽), and glycylglycyl-β-2-thienyl-DL-alanine (●) for *Escherichia coli* in the presence of glycylglycyl-DL-phenylalanine (30 units per 5 ml.).

β-2-thienyl-DL-alanylglycine were more toxic at a concentration of 300 units per 5 ml. than the other compounds were at 10,000 units per 5 ml. The results indicate that glycylglycyl-β-2-thienyl-DL-alanine and glycyl-β-2-thienyl-DL-alanylglycine prevent the utilization of glycylglycyl-DL-phenylalanine in a way that cannot be attributed to any of the possible hydrolytic products such as glycyl-β-2-thienylalanine or β-2-thienylalanine. It also is evident that the order of attachment of the amino acids in the tripeptide affected utilization since β-2-thienyl-DL-alanylglycylglycine was much less toxic than the other two isomeric tripeptides.

The greater toxicity manifested by glycylglycyl-β-2-thienyl-DL-alanine in the presence of glycylglycyl-DL-phenylalanine does not appear to be a synergistic effect of the possible hydrolytic products, β-2-thienylalanine and glycyl-β-2-thienylalanine. In another test, not shown in Fig. 5, in which 300 units of glycylglycyl-β-2-thienyl-DL-alanine produced complete inhibition of growth in the presence of 30 units of glycylglycyl-DL-phenylalanine, a mixture of 3000 μg. of β-2-thienyl-DL-alanine and 3000 units of glycyl-β-2-thienyl-DL-alanine (equivalent to 6000 μg. of β-2-thienyl-DL-alanine) was required for the same inhibition.

In the presence of DL-phenylalanylglycylglycine (30 units per 5 ml.), the analogous tripeptide β-2-thienyl-DL-alanylglycylglycine produced complete growth inhibition at a concentration of 500 units per 5 ml.; however, free β-2-thienyl-DL-alanine, glycyl-β-2-thienyl-DL-alanine, and β-2-thienyl-DL-alanylglycine failed to inhibit growth completely even at much higher concentrations (Fig. 6). β-2-Thienyl-DL-alanylglycylglycine was more toxic in the presence of DL-phenylalanylglycylglycine than in the presence of glycylglycyl-DL-phenylalanine or glycyl-DL-phenylalanylglycine (*cf.* Figs. 5, 6, and 7). Glycylglycyl-β-2-thienyl-DL-alanine and glycyl-β-2-thienyl-DL-alanylglycine were more toxic than β-2-thienyl-DL-alanylglycylglycine under all of the conditions employed.

A striking example of peptide utilization was found in a comparison of the toxicity of glycylglycyl-β-2-thienyl-DL-alanine and glycyl-β-2-thienyl-DL-alanylglycine with that of β-2-thienyl-DL-

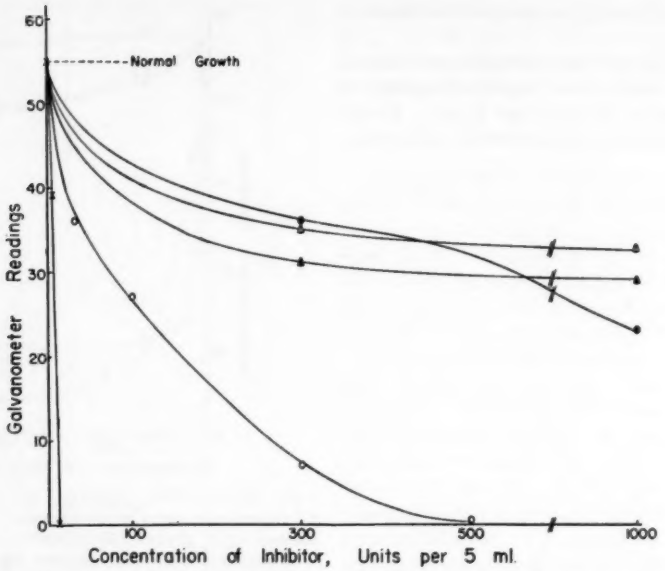

FIG. 6. Relative toxicity of glycylglycyl-β-2-thienyl-DL-alanine (×), glycyl-β-2-thienyl-DL-alanylglycine (×), β-2-thienyl-DL-alanylglycylglycine (○), glycyl-β-2-thienyl-DL-alanine (▲), β-2-thienyl-DL-alanylglycine (●), and β-2-thienyl-DL-alanine (△) for *Escherichia coli* in the presence of DL-phenylalanylglycylglycine (30 units per 5 ml.).

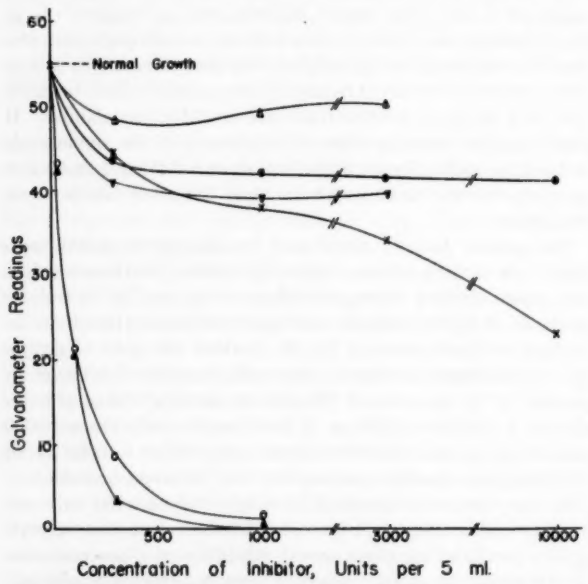

FIG. 7. Relative toxicity of glycylglycyl-β-2-thienyl-DL-alanine (○), glycyl-β-2-thienyl-DL-alanylglycine (▲), β-2-thienyl-DL-alanylglycylglycine (△), β-2-thienyl-DL-alanine (×), glycyl-β-2-thienyl-DL-alanine (●), and β-2-thienyl-DL-alanylglycine (▽) for *Escherichia coli* in the presence of glycyl-DL-phenylalanylglycine (30 units per 5 ml.).

alanine in the presence of DL-phenylalanylglycylglycine (30 units per 5 ml.). Whereas growth was completely prevented by 10 units of glycylglycyl-β-2-thienyl-DL-alanine or glycyl-β-2-thienyl-DL-alanylglycine (Fig. 6), 10,000 μg. of β-2-thienyl-DL-alanine were required to prevent growth.

Further evidence of competition between tripeptides of similar structure is seen in Fig. 7 where the toxicity of β-2-thienyl-DL-alanine and its peptides is compared in the presence of glycyl-DL-phenylalanylglycine. Here again glycyl-β-2-thienyl-DL-alanylglycine and glycylglycyl-β-2-thienyl-DL-alanine were much more toxic than free β-2-thienyl-DL-alanine, β-2-thienyl-DL-alanylglycine, glycyl-β-2-thienyl-DL-alanine, or β-2-thienyl-DL-alanylglycylglycine.

When tested in the presence of L-leucyl-L-phenylalanylglycine, the analogous L-leucyl-β-2-thienyl-L-alanylglycine was more toxic than L-leucyl-β-2-thienyl-L-alanine, β-2-thienyl-DL-alanylglycine, or β-2-thienyl-DL-alanine (Fig. 8). Glycylglycyl-β-2-thienyl-DL-alanine and glycyl-β-2-thienyl-DL-alanylglycine were slightly more toxic than L-leucyl-β-2-thienyl-L-alanylglycine under these conditions.

The toxicity of the tripeptides of β-2-thienylalanine was reversed noncompetitively by phenylalanine and its dipeptides. Addition of 30 μg. of DL-phenylalanine per 5 ml., or the equivalent concentration of glycyl-DL-phenylalanine or DL-phenylalanylglycine, stimulated growth of *E. coli* in the presence of 10,000 units of glycylglycyl-β-2-thienyl-DL-alanine or glycyl-β-2-thienyl-DL-alanylglycine.

Previous work has demonstrated inhibition of separate sites of utilization of phenylalanine, phenylpyruvic acid, and glycylphenylalanine by the analogous thiophene derivatives (23). The present work indicates that utilization of a tripeptide by *E. coli* may occur without previous hydrolysis and that the site of utilization can be blocked in a specific way by a tripeptide analogue. The data in Table II support this conclusion. When the medium was supplemented with a mixture of comparatively high levels of glycylglycyl-DL-phenylalanine, glycyl-DL-phenylalanine, glycylglycyl-β-2-thienyl-DL-alanine, and glycyl-β-2-thienyl-DL-alanine, resulting in growth inhibition, addition of as little as 10 μg. of DL-phenylalanine stimulated growth. The

effect of DL-phenylalanine was nullified by 500 μg. of β-2-thienyl-DL-alanine. When growth was inhibited in a medium containing DL-phenylalanine, glycylglycyl-DL-phenylalanine, β-2-thienyl-DL-alanine, and glycylglycyl-β-2-thienyl-DL-alanine, the growth stimulating effect of a low level of glycyl-DL-phenylalanine was blocked by a relatively low level of glycyl-β-2-thienyl-DL-alanine. When the medium was supplemented with DL-phenylalanine, glycyl-DL-phenylalanine, β-2-thienyl-DL-alanine, and glycyl-β-2-thienyl-DL-alanine at levels which prevented growth of *E. coli*, addition of 3 units of glycylglycyl-DL-phenylalanine stimulated growth, and the effect of glycylglycyl-DL-phenylalanine was almost completely nullified by 30 units of glycylglycyl-β-2-thienyl-DL-alanine. Thus, 3 units of the tripeptide of phenylalanine (glycylglycyl-DL-phenylalanine) stimulated growth in the presence of 100 units of its possible hydrolytic products (50 μg. of DL-phenylalanine and 50 units of glycyl-DL-phenylalanine), and 30 units of the tripeptide of β-2-thienylalanine (glycylglycyl-β-2-thienyl-DL-alanine) inhibited growth in the presence of 6000 units of its possible hydrolytic products (3000 μg. of β-2-thienyl-DL-alanine and 3000 units of glycyl-β-2-thienyl-DL-alanine). It appears reasonable to conclude that phenylalanine and its analogue compete at one site, the dipeptide and its analogue at another site, whereas the tripeptide of phenylalanine and its analogue compete at a third site.

Since the isomeric tripeptides of β-2-thienylalanine did not behave in the same manner under all conditions, the order of attachment of the amino acids in a tripeptide appears to influence the mode of utilization. The tripeptide in which β-2-thienylalanine was the first moiety (β-2-thienyl-DL-alanylglycylglycine) was less toxic than the tripeptide in which it was the second moiety (glycyl-β-2-thienyl-DL-alanylglycine) or the third moiety (glycylglycyl-β-2-thienyl-DL-alanine) under a variety of testing conditions.

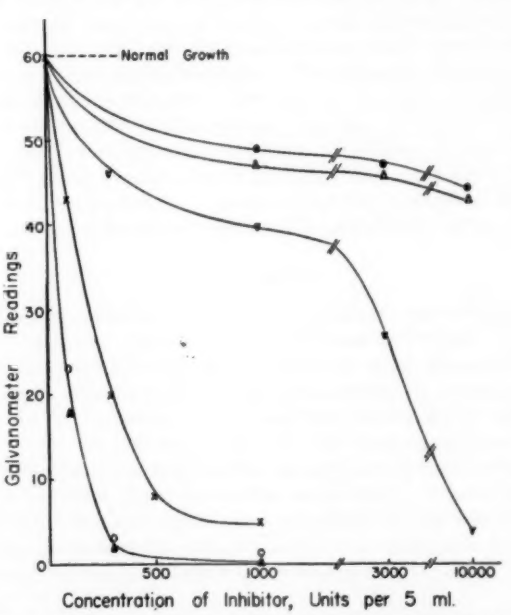

FIG. 8. Relative toxicity of L-leucyl-β-2-thienyl-L-alanylglycine (×), L-leucyl-β-2-thienyl-L-alanine (●), β-2-thienyl-DL-alanylglycine (△), glycylglycyl-β-2-thienyl-DL-alanine (○), glycyl-β-2-thienyl-DL-alanylglycine (▲), and β-2-thienyl-DL-alanine (▽) for *Escherichia coli* in the presence of L-leucyl-L-phenylalanylglycine (30 units per 5 ml.).

TABLE II

Inhibition of separate sites of utilization of phenylalanine, glycylphenylalanine and glycylglycylphenylalanine

Test organism, *E. coli* strain 9723; incubated for 18 hours at 37°.

GGPA* 30; GPA, 30; GGTA, 2000; GTA, 3000			PA, 30; GGPA 30; TA, 3000; GGTA, 2000			PA, 50; GPA, 50; TA, 3000; GTA, 3000		
PA†	TA†	Galvanometer readings‡	GPA†	GTA†	Galvanometer readings‡	GGPA†	GGTA†	Galvanometer readings‡
0	0	0	0	0	3	0	0	4
3	0	23	1	0	19	1	0	36
10	0	46	3	0	32	3	0	44
10	100	36	10	0	45	3	10	28
10	300	12	10	100	44	3	30	6
10	500	2	10	300	5	3	100	3

* The abbreviations are as follows: GGPA, glycylglycyl-DL-phenylalanine; GPA, glycyl-DL-phenylalanine; PA, DL-phenylalanine; GGTA, glycylglycyl-β-2-thienyl-DL-alanine; GTA, glycyl-β-2-thienyl-DL-alanine; TA, β-2-thienyl-DL-alanine.

† Units per 5 ml.; 1 unit is equivalent to 1 μg. of DL-phenylalanine or 1 μg. of β-2-thienyl-DL-alanine.

‡ A measure of culture turbidity; distilled water reads 0 and an opaque object reads 100.

The point at which competition between analogous peptides occurs is not shown by the present study, but the data indicate that tripeptides can function in the growth of *E. coli* in a manner that is independent of conversion to the corresponding dipeptides or free amino acids. If permeability of the cell wall is an important factor, as some studies indicate (24, 25), and the tripeptides are competing for entrance into the cell or some compartment of the cell, one must conclude that permeation is a very specific process, with separate points of entrance for amino acids, keto acids, dipeptides, and tripeptides.

Although a high concentration of resting cells of *E. coli* caused hydrolysis of tripeptides of β-2-thienylalanine, this process does not account for the effects of these tripeptides upon the growth of *E. coli* in the presence of various phenylalanine peptides. Any effect of hydrolytic products was "diluted out" when the tripeptides were tested in the presence of high levels of hydrolytic products, as reported in Table II. If hydrolysis of the peptides to form β-2-thienylalanine were an essential preliminary step in the utilization of the tripeptides, one might expect glycyl-β-2-thienyl-DL-alanylglycine (in which β-2-thienyl-DL-alanine is "covered" by two peptide bonds) to be less active than either glycylglycyl-β-2-thienyl-DL-alanine or β-2-thienyl-DL-alanylglycylglycine. Since glycyl-β-2-thienyl-DL-alanylglycine was as active as glycylglycyl-β-2-thienyl-DL-alanine in various tests, one might reasonably assume that the peptide is functioning as a unit or that the β-2-thienylalanine within the peptides is readily converted directly into an active form.

SUMMARY

Preparations are reported for glycylglycyl-β-2-thienyl-DL-alanine, glycyl-β-2-thienyl-DL-alanylglycine, β-2-thienyl-DL-alanylglycylglycine, L-leucyl-β-2-thienyl-L-alanine, L-leucyl-β-2-thienyl-L-alanylglycine, L-leucyl-L-phenylalanylglycine, and various inter-

mediates leading to these peptides. Resolution of β-2-thienyl-DL-alanine by the papain method is described.

Tripeptides of phenylalanine were more active than phenylalanine in reversing the toxicity of β-2-thienylalanine in *Escherichia coli* strain 9723. Tripeptides of β-2-thienylalanine were comparable with free β-2-thienylalanine as growth inhibitors for *E. coli* when tested in the salts-glucose medium, and when tested in the presence of analogous tripeptides of phenylalanine they were many times as toxic as free β-2-thienylalanine. The toxicity of the tripeptides of β-2-thienylalanine was reversed noncompetitively by phenylalanine and its dipeptides. The results are interpreted as indicating that tripeptides of β-2-thienylalanine compete with tripeptides of phenylalanine in a specific way and that their route of utilization is independent of the route followed by the possible hydrolytic products.

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REFERENCES

1. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169**, 91 (1947).
2. Dunn, F. W., and Dittmer, K., *J. Biol. Chem.*, **188**, 263 (1951).
3. Kihara, H., and Snell, E. E., *J. Biol. Chem.*, **212**, 83 (1955).
4. Dunn, F. W., Ravel, J. M., and Shive, W., *J. Biol. Chem.*, **219**, 809 (1956).
5. Ifland, P. W., Ball, E., Dunn, F. W., and Shive, W., *J. Biol. Chem.*, **230**, 897 (1958).
6. Simmonds, S., and Fruton, J. S., *J. Biol. Chem.*, **180**, 635 (1949).
7. Hirsch, M. L., and Cohen, G. N., *Biochem. J.*, **53**, 25 (1953).
8. Dunn, F. W., Humphreys, J., and Shive, W., *Arch. Biochem. Biophys.*, **71**, 475 (1957).
9. Fox, S. W., and Pettinga, C. W., *Arch. Biochem. Biophys.*, **25**, 13 (1950).
10. Fox, S. W., Pettinga, C. W., Halverson, J. S., and Wax, H., *Arch. Biochem. Biophys.*, **25**, 21 (1950).
11. Albertson, N. F., *J. Am. Chem. Soc.*, **73**, 452 (1951).
12. Huang, H. T., and Niemann, C., *J. Am. Chem. Soc.*, **73**, 475 (1951).
13. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **180**, 193 (1949).
14. Sheehan, J. C., and Frank, V. S., *J. Am. Chem. Soc.*, **71**, 1856 (1949).
15. Albertson, N. F., *J. Am. Chem. Soc.*, **72**, 1396 (1950).
16. Vaughan, J. R., Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).
17. Albertson, N. F., and McKay, F. C., *J. Am. Chem. Soc.*, **75**, 5323 (1953).
18. Crowe, B. F., and Nord, F. F., *J. Org. Chem.*, **15**, 688 (1950).
19. Dunn, F. W., *J. Org. Chem.*, **21**, 1525 (1956).
20. Ferger, M. F., and du Vigneaud, V., *J. Biol. Chem.*, **174**, 241 (1948).
21. Williams, R. J., McAlister, E. D., and Roehm, R. R., *J. Biol. Chem.*, **83**, 315 (1929).
22. Dunn, F. W., *J. Biol. Chem.*, **227**, 575 (1957).
23. Dunn, F. W., Ravel, J. M., Mollenhauer, B. F., and Shive, W., *J. Biol. Chem.*, **233**, 959 (1956).
24. Rowlands, D. A., Gale, E. F., Folkes, J. P., and Marrian, D. H., *Biochem. J.*, **65**, 519 (1957).
25. Mitchell, P., and Moyle, J., *Symposium Soc. Gen. Microbiol.*, **6**, 150 (1956).

Glutamic Dehydrogenase

I. THE EFFECT OF COENZYME ON THE SEDIMENTATION VELOCITY AND KINETIC BEHAVIOR*

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It has been reported that high concentrations of reduced diphosphopyridine nucleotide as well as 1,10(ortho)-phenanthroline will cause dissociation of crystalline beef liver glutamic dehydrogenase as observed by the sedimentation behavior of the enzyme in the ultracentrifuge (1). Although this enzyme undergoes dissociation upon dilution (2), the effect of DPNH or 1,10-phenanthroline may be obtained at enzyme concentrations at which there should be essentially no dissociation at all. This result suggested that the extent of association or dissociation of the enzyme might play a role in the catalytic function of the enzyme. The purpose of the present investigation, then, was to study the effects of the different coenzymes for the reaction on the sedimentation and kinetic behavior and to determine the relationship between initial velocities of the enzymatic reaction obtained kinetically and the sedimentation behavior as observed in the ultracentrifuge. It will be shown in this paper that the coenzymes DPN, DPNH, TPN and TPNH, all of which are active with glutamic dehydrogenase, influence the sedimentation behavior of the enzyme at levels which are identical with the coenzyme concentrations used in the determination of the kinetic constants. Consequently, the rates of the enzymatic reaction are determined by the degree of association or dissociation of the enzyme. The data presented in this paper show that the enzyme, when dissociated, is inactive. The kinetic and sedimentation data also show that the active site for all the coenzymes is the same, but that in addition, DPN and DPNH are bound to a second, noncatalytically active, site.

EXPERIMENTAL

The experiments to be described in this paper were carried out in Tris[1]-acetate buffer at pH 7.9 to 8.0 (measured at 25°).

Sedimentation Measurements—Sedimentation experiments were conducted with the Spinco model E analytical ultracentrifuge[2] with the use of two double sector cells at a speed of 42,040 r.p.m. The temperature of the rotor in most cases was considered to be the average of the temperature readings directly before and after the sedimentation experiment. In some later sedimentation experiments the temperature of the rotor was accurately determined with an RTIC unit manufactured by Spinco. All the sedimentation experiments were made in Tris-acetate buffer which was 0.08 M with respect to acetate and 5×10^{-4} M with respect to Versene (ethylenediaminetetraacetate). Versene was used in order to chelate contaminating heavy metals which inhibit the enzymatic reaction. The amount of Versene used did not influence the extent of dissociation of enzyme. The sedimentation coefficients were corrected to 20° for standardization, but corrections for viscosity changes due to the addition of coenzyme or other compounds were not made, since the concentrations of these compounds were relatively small compared to the concentration of buffer. The results in this paper are presented as s_{20} values, not corrected to water.

Kinetic Measurements—Kinetic experiments were performed with a Beckman DU spectrophotometer coupled to a Brown recorder with which 80 to 100 per cent transmission could be expanded to full scale (11 inches). The recorder has a pen speed of 1 second per full scale deflection and the paper moved at the rate of 4 inches per minute. Measurements were usually made within the first few per cent of the reaction. For experiments involving low concentrations of DPN as coenzyme, cuvettes of 10-cm. optical path, holding a total volume of 25 ml. were used to increase sensitivity. With DPNH as coenzyme, cuvettes of only 1-cm. optical path were used and the slit width was normally held at less than 0.30 mm. It was sometimes necessary to use wave lengths higher that 340 mμ to accomplish this.

For DPN as substrate, all experiments were carried out in 0.01 M Tris-acetate, pH 8, at a glutamate concentration of 0.05 M. For DPNH as substrate all experiments were carried out in the same buffer, at an α-ketoglutarate concentration of 0.05 M and an NH_4Cl concentration of 0.1 M. The standard assay solution used was 1×10^{-4} M with respect to DPNH.

In all kinetic experiments, some Versene was added either to the cuvette or to the stock enzyme solution. The final concentration of Versene in the reaction mixture was 1×10^{-4} M or less. The presence of these low concentrations of Versene increased the initial velocity, presumably as a result of the removal of heavy metal impurities in the enzyme preparation. However, the concentrations of Versene used were considerably lower than those observed to affect the kinetic parameters of the enzymatic reaction.

Enzyme—Crystalline glutamic dehydrogenase from beef liver was obtained as a suspension of crystals in $(NH_4)_2SO_4$ from C. F. Boehringer, Germany. The enzyme is identical in every way with enzyme prepared by the method of Strecker (3) and recrystallized with Na_2SO_4 (2), except that the ratio of optical density at 280 mμ to 260 mμ was about 1.8. Under assay conditions, the turnover number of the enzyme was 40,000 $min.^{-1}$ based on the molecular weight of 1×10^6. When the initial

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[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

[2] The author would like to thank Miss Carmelita Lowry for performing the sedimentation velocity experiments.

velocity under normal assay conditions is extrapolated to infinite DPNH concentration in the presence of ADP (to prevent inhibition), the turnover number is calculated to be as high as 170,000 min.$^{-1}$. Enzyme used for kinetic experiments was kept at 0° in 0.1 M Tris-acetate, pH 8.0, in the presence of a small amount of Versene. Normally, this enzyme underwent very little denaturation for periods up to about 2 hours. When the enzyme was to be used for sedimentation experiments, the crystals were centrifuged at high speed, the supernatant fluid poured off, and the packed crystals washed several times with cold buffer. The crystals were then dissolved in the buffer to be used in the sedimentation experiment.

Reagents—DPN, DPNH, TPN, and TPNH were obtained either from the Sigma Chemical Company or Pabst Laboratories. These compounds were about 90 to 95 per cent pure. α-Ketoglutarate was obtained from Nutritional Biochemicals Corporation and was recrystallized from acetone-benzene mixtures before use. The sodium-L-glutamate used was over 99 per cent pure.

RESULTS

Kinetic Behavior of Enzyme at Constant pH—Initial velocities as a function of coenzyme concentration have been obtained at pH 8.0 in 0.01 M Tris-acetate buffer. In all cases, the concentration of α-ketoglutarate, NH_4^+, or glutamate was held at constant and noninhibitory levels.

Initial velocity experiments with DPN used as coenzyme show that high concentrations of DPN yield velocities greater than would be expected by extrapolation from velocities at low DPN concentration. Such results with DPN have been observed previously (4). Fig. 1 shows the variation of reciprocal initial velocity as a function of reciprocal DPN concentration at 10.5° in 0.01 M Tris-acetate, pH 8.0, at a glutamate concentration of 0.05 M. These data may be represented by the equation

$$v = V_1 \left[1 + \frac{V_2(\text{DPN})}{V_1 K_2}\right] \Big/ \left[1 + \frac{(\text{DPN})}{K_1} + \frac{K_2}{(\text{DPN})}\right] \quad (1)$$

where V_1 and V_2 are the maximal velocities in the absence and presence of excess DPN at a particular glutamate concentration. The solid line drawn in Fig. 1 was constructed on the assumption that $K_1 = 1.4 \times 10^{-5}$ M, $K_2 = 2.5 \times 10^{-4}$ M and $V_2/V_1 = 3.0$.

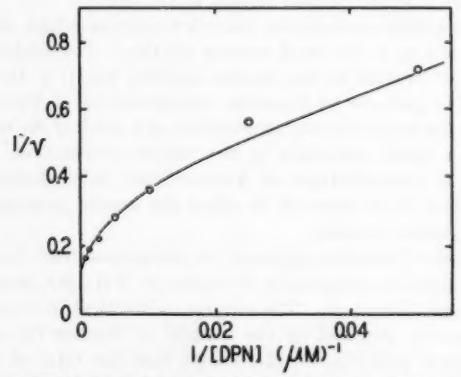

FIG. 1. Plot of reciprocal initial velocity against reciprocal DPN concentration at pH 8.0 in 0.01 M Tris-acetate buffer and 10.5°. Solid line is drawn from Equation 1 and values given in the text.

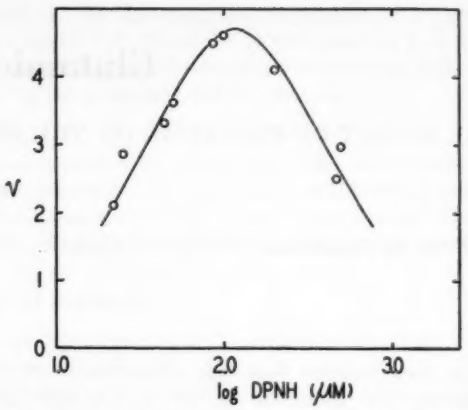

FIG. 2. Plot of the initial velocity for DPNH oxidation as a function of log of the DPNH concentration, at pH 8.0, in 0.01 M Tris-acetate buffer and 25°. α-Ketoglutarate and NH_4Cl concentrations are 0.05 M and 0.1 M, respectively. Solid line is drawn from Equation 2 and values given in text.

At lower DPN concentrations, the plot is linear with the points falling on the line drawn. These lower concentrations have been omitted in Fig. 1 in order to show clearly the substrate activation.

At 25° the data for the reduction of DPN also obey Equation 1. Under these conditions, $K_1 = 7 \times 10^{-5}$ M, $K_2 = 2 \times 10^{-3}$ M and V_2/V_1 is about 4. Thus, there is a 5-fold increase in K_1, and an 8-fold increase in K_2 for an increase in temperature of only 15°. Relations of the type represented by Equation 1 may be derived by the usual steady state methods for two different mechanisms. (*a*) The first assumes that the binding of a 2nd molecule of DPN increases the rate of reduction of the 1st molecule of DPN, *i.e.* that there is both an active and an activating site for DPN. (*b*) The second assumes that there are two active sites for DPN which are either different and independent or identical but interact in pairs (see, for example, (5 and 6)). Thus, K_1 in Equation 1 is the Michaelis constant for DPN, but K_2 may be either a Michaelis constant or a dissociation constant. From results which are to be presented in the following paper (7), the one of these two mechanisms which assumes both an active and activating site for DPN appears to be correct.

Initial velocity measurements have been made with the use of DPNH at 25°, pH 8.0, an 0.01 M Tris-acetate buffer. It has been found that DPNH is a strong inhibitor of the enzymatic reaction at concentrations above 1×10^{-4} M. The results are plotted in Fig. 2 as initial velocity as a function of log DPNH concentration. The data are found to fit the equation

$$v = V' \Big/ \left[1 + \frac{(\text{DPNH})}{K_{\text{DPNH}}} + \frac{K'_{\text{DPNH}}}{(\text{DPNH})}\right] \quad (2)$$

which is derived for a mechanism that provides that the addition of a 2nd molecule of DPNH at or near the active site for DPNH will prevent the enzymatic reaction from proceeding. In Equation 2, K_{DPNH} and K'_{DPNH} are the Michaelis and inhibition constants for DPNH, respectively, and V' is a constant related to the velocity under the conditions of constant α-ketoglutarate and NH_4^+ concentration. The solid line in Fig. 2 has been drawn with the use of Equation 2 with $K_{\text{DPNH}} = 1.15 \times 10^{-4}$ M and $K'_{\text{DPNH}} = 1.25 \times 10^{-4}$ M. The calculation of these constants was made in a manner identical to the calculation of

ionization constants from a symmetrical, bell-shaped plot of maximal velocity against pH (8). The fact that the Michaelis and inhibition constants are nearly the same, however, leads to a fairly large possible error in an accurate determination of these constants. The interpretation of two binding sites for DPNH, only one of which is active, is entirely consistent with the results for DPN reduction, provided that the results for DPN may be explained by a mechanism which assumes both an active and an activating site. The affinity of the enzyme for the 2nd molecule of DPNH, however, appears to be considerably higher than for the activating molecule of DPN. As opposed to the results for DPN, experiments at 14° yield Michaelis and inhibition constants for DPNH almost identical to those at 25°.

The Michaelis constants for TPN and TPNH have been determined at 25° under the same conditions used for DPN and DPNH. Fig. 3 shows a reciprocal plot of the data for TPN. This plot shows no deviation from linearity and thus no indication of substrate activation or inhibition. Similar results are obtained with TPNH. The Michaelis constants for TPN and TPNH are 0.95×10^{-4} M and 0.35×10^{-4} M, respectively. The data indicate that in contrast to DPN or DPNH, a 2nd molecule of the triphosphopyridine nucleotide is not bound to a site which is near the active site for these coenzymes, indicative of a certain specificity for the adenosine 5′-phosphate and not for the adenosine 2′-phosphate.

Specificity of Active Site for Coenzyme—In order to be able to correlate results obtained with the di- and triphosphopyridine nucleotides, it is necessary to determine the specificity of the active site. To do this, both DPN and TPN have been used to inhibit the oxidation of DPNH and the oxidation of TPNH. Fig. 4 shows results obtained with TPNH as substrate and either DPN or TPN as inhibitor. The data are plotted as reciprocal velocity against reciprocal TPNH concentration. A similar procedure has been used by Hayes and Velick to show that DPN and DPNH are bound to the same site of yeast alcohol dehydrogenase (9), but in addition, Alberty has shown that for most two-substrate enzyme mechanisms, inhibition constants calculated with use of product coenzyme as inhibitor are true dissociation constants provided that the product coenzyme binds the free enzyme (10). The inhibition constant for TPN calculated from Fig. 4 is 1.2×10^{-4} M. The difference between the Michaelis and inhibition constants is most probably due to the fact that the inhibition constant is determined at considerably higher salt concentration (0.1 M NH_4Cl) which tends to increase

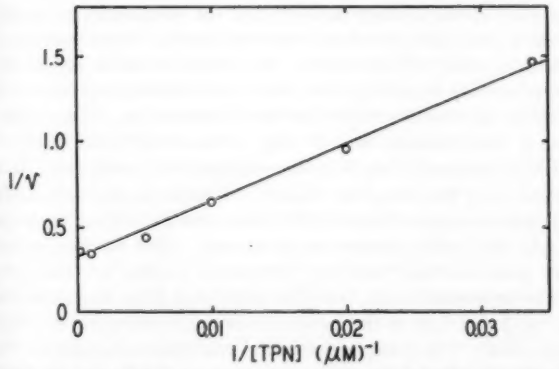

FIG. 3. Reciprocal plot for the reduction of TPN, at pH 8.0, in 0.01 M Tris-acetate at 25°. Glutamate concentration was 0.05 M.

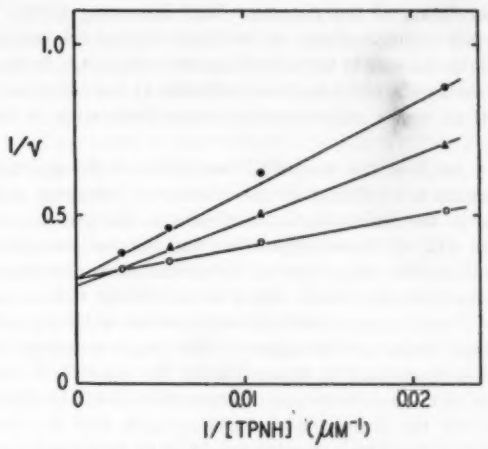

FIG. 4. The inhibitory effects of TPN (●——●) and DPN (△——△) on the enzymatic oxidation of TPNH. TPN concentration, 3.5×10^{-4} M; DPN concentration, 4×10^{-4} M; ○——○ is for TPNH alone at a concentration of α-ketoglutarate and NH_4Cl of 0.05 M and 0.1 M, respectively. Results obtained at 25°, pH 8.0, in 0.01 M Tris-acetate.

TABLE I

Inhibition constants of DPN and TPN

Coenzyme	Inhibitor	Inhibition constant	Michaelis constant of inhibitor
DPNH.....	TPN	1.0×10^{-4} M	0.95×10^{-4} M
TPNH.....	TPN	1.2×10^{-4} M	0.95×10^{-4} M
DPNH.....	DPN	1.3×10^{-4} M	0.7×10^{-4} M
TPNH.....	DPN	1.3×10^{-4} M	0.7×10^{-4} M

the Michaelis constant. The inhibition by DPN as shown in Fig. 4 is not quite competitive. The concentration of DPN used was 4×10^{-4} M, and there appears to be activation as a result of DPN-binding to an activating site. At lower concentrations, the inhibition by DPN is strictly competitive. Results from experiments conducted at constant TPNH concentration and varying DPN concentration are consistent with a mechanism which assumes that the binding of DPN to the TPNH site will give competitive inhibition, but that the binding of DPN to the second site will cause activation.

Similar experiments have been carried out with DPN and TPN used as inhibitors for the oxidation of DPNH with similar results. The competitive inhibition constants for DPN and TPN in the oxidation of DPNH or TPNH as compared to their Michaelis constants are listed in Table I. These results eliminate the possibility that the active sites for DPNH and TPNH are independent of each other. The fact that the inhibition constants for DPN or TPN, are essentially the same regardless of whether DPNH or TPNH is used as coenzyme shows that the active site for DPNH is indeed the active site for TPNH also. Further interpretation of the data is made on this basis.

Effect of Coenzyme on Sedimentation Velocity Behavior—Since the enzyme undergoes reversible dissociation, the sedimentation coefficient will be dependent on the enzyme concentration (2). However, at constant enzyme concentration, it is found that the coenzymes of the enzymatic reaction all have some effect on the sedimentation velocity behavior and therefore on the extent

of dissociation of the enzyme. With increasing DPN, TPN, or TPNH concentrations, at constant enzyme concentrations, there is an increase in the sedimentation coefficient. In addition to increasing the sedimentation coefficient at low concentrations, DPNH at higher concentrations causes dissociation of the enzyme.

As a result of the reversible dissociation of the enzyme, the sedimentation coefficient in the absence of coenzyme and the change in the sedimentation coefficient in the presence of coenzyme will, of course, depend on the enzyme concentration. Fig. 5 shows the dependence of the sedimentation coefficient on DPN concentration (dark circles) at an average rotor temperature of 9° and constant enzyme concentration of 1.0 mg. per ml. The open circles and triangles of this graph represent initial velocities determined at approximately the same DPN concentrations as the sedimentation experiments and will be discussed below. In the absence of coenzyme, s_{20} is 14.6 S. On the assumption that the molecular weight of undissociated enzyme is 1×10^6 (2) and of dissociated enzyme, 2.5×10^5 (1), then at the concentration of enzyme used in the experiment shown in Fig. 5, the enzyme is over 85 per cent dissociated.

Increasing concentrations of both TPN and TPNH also increase the sedimentation coefficient of the enzyme. However, the extent of increase in the sedimentation coefficient does not appear to be quite as great as with DPN. Experiments with TPN or TPNH concentrations of less than 10^{-4} M indicate that the maximal sedimentation coefficient reached is about 26 S. Higher TPN or TPNH concentrations do not have much effect on the sedimentation coefficient. Thus, from 1×10^{-4} to 6×10^{-4} M TPNH, there is an increase of only 0.5 S, whereas Fig. 5 shows an increase of almost 2 S over this concentration range when DPN is used. Yet, the Michaelis constants for DPN and TPNH at this pH and temperature are almost identical.

The variation of the sedimentation coefficient with DPNH concentration is quite different from that for DPN, TPN, or TPNH. Results obtained with increasing DPNH concentrations, constant enzyme concentration, and constant temperature are shown in Fig. 6. For these experiments, the enzyme concentration was 3 mg. per ml., and the sedimentation coefficient in the absence of DPNH was found to be 22.9 S. Fig. 6 shows that increasing DPNH concentration leads to an initial increase in the sedimentation coefficient to a maximum of 26.4 S at slightly over 1×10^{-4} M DPNH. At higher concentrations of DPNH there is a slight decrease in the value of the sedimentation coefficient and above a concentration of about 3.5×10^{-4} M, two peaks with remarkably different sedimentation coefficients appear in the sedimentation pattern. The approximate areas of these two peaks at the higher DPNH concentrations are shown in Fig. 6 in parentheses above the points showing the value of the sedimentation coefficients. The concentration of protein as represented by the area of the two peaks is subject to considerable error as a result of the fast moving component sedimenting in the presence of a slower moving component and as a result of the dissociation of enzyme upon dilution, although these errors tend to cancel out. It will be shown below that the dissociation of enzyme by high DPNH concentrations is directly related to the inhibition of the initial velocity of the enzymatic reaction at high DPNH concentrations.

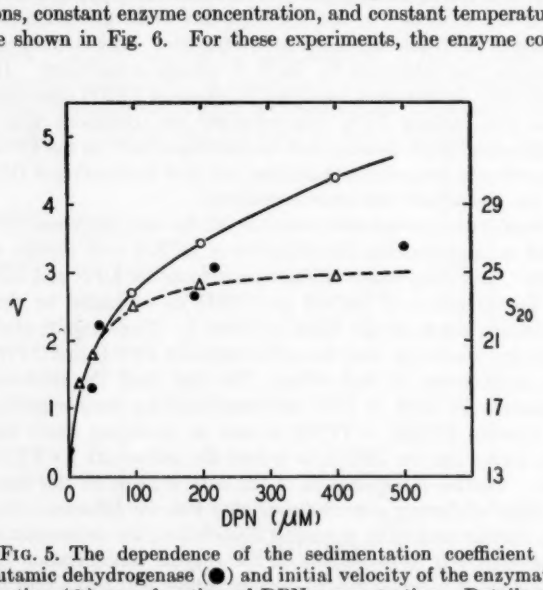

FIG. 5. The dependence of the sedimentation coefficient of glutamic dehydrogenase (●) and initial velocity of the enzymatic reaction (○) as a function of DPN concentration. Details are given in the text.

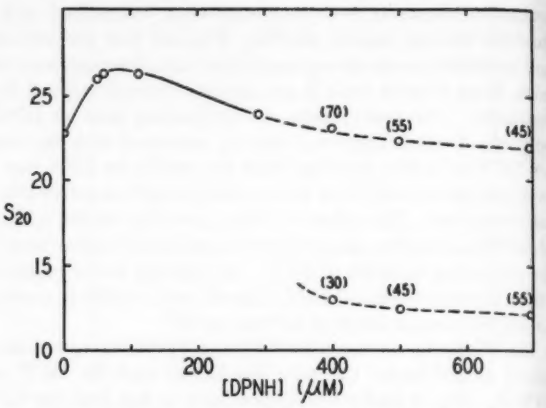

FIG. 6. The dependence of the sedimentation coefficient of glutamic dehydrogenase as a function of DPNH concentration. Numbers in parentheses above points at high DPNH concentrations are approximate areas of the two peaks. Experiments performed at an average rotor temperature of 9° and enzyme concentration of 3.0 mg. per ml.

DISCUSSION

Correlation of Kinetic and Sedimentation Data—The results obtained above strongly indicate that the association of the enzyme is intimately involved in the mechanism of the enzymatic reaction, since concentrations of coenzyme which affect the sedimentation behavior of the enzyme are similar to those which must be used to determine the kinetic constants. This conclusion is best demonstrated by Fig. 5 which shows the effect of DPN concentration on both the sedimentation coefficient (dark circles) and the reaction velocity. Results at approximately the same concentrations of DPN have been plotted for both the kinetic and sedimentation measurements. The reaction velocities were obtained from the plot shown in Fig. 1. The open circles represent initial velocities calculated from the solid line in Fig. 1 and include the increase in initial velocity due to DPN activation. The triangles are initial velocities calculated on the assumption that there is no activation by DPN, *i.e.* that the

Lineweaver-Burk plot is linear with a Michaelis constant of 1.4×10^{-5} M. The plots of s_{20} and initial velocity coincide only if it is assumed that binding of DPN to the active site of the enzyme causes association of the enzyme molecule. In the sedimentation experiments, the enzyme is only about 85 per cent dissociated in the absence of DPN, contrasted to 100 per cent dissociation in initial velocity experiments. However, the plot of s_{20} against DPN is largely dependent on the dissociation constant of the enzyme-DPN complex and independent of the fact that the absolute concentration of the enzyme used in the sedimentation experiments is considerably higher than that used in the initial velocity experiments. The shape of the plot of initial velocity as a function of DPN concentration is, of course, dependent only on the values of the Michaelis and activation constants or, as in the broken line of Fig. 5, on the Michaelis constant alone.

Similar experiments with other coenzymes give similar results. Such experiments indicate that it is coenzyme binding with the active site which is responsible for the majority of the change of the sedimentation coefficient. Similarly, it is apparent that the binding of DPN to the nonactive site has only a small effect on the value of the sedimentation coefficient. It is therefore quite clear that a comparison of sedimentation coefficients and initial velocities as a function of coenzyme concentration is valid, and that there is a direct correlation between initial velocity and degree of association of the enzyme. The correlation as shown in Fig. 5 indicates that the Michaelis constant for DPN determined kinetically is a true dissociation constant. This conclusion is supported by the fact that the Michaelis constant for DPN, in the presence of ADP to prevent DPN activation (7), does not vary with glutamate concentration, a result which also indicates that the experimentally determined Michaelis constant is a dissociation constant (11).

As pointed out above, the Michaelis and inhibition constants for DPNH do not appear to change much between 14–25°. Thus it is possible to make a rough correlation between DPNH inhibition and the areas of the two peaks in the sedimentation experiments. For example, Fig. 2 shows that at 4×10^{-4} M DPNH, the initial velocity is only about 70 per cent of the maximal initial value at 1.2×10^{-4} M DPNH. Fig. 6 shows that at this DPNH concentration the area of the fast sedimenting peak is about 70 per cent of the total. Similar results are obtained at the higher DPNH concentrations. Such results are, of course, only very approximate.

Many other such correlations may be made, all of which show the close relation between sedimentation and kinetic behavior. These correlations show that the degree of association or dissociation directly determines initial velocities or, alternatively, that the reaction velocities are a measure of the state of association or dissociation of the enzyme molecule. Clearly, the association-dissociation equilibrium must be considered in the interpretation of kinetic results obtained for this enzyme.

Binding Sites for Coenzyme—The present studies do not indicate how many catalytically active sites there are in the enzyme molecule. However, it is interesting to note that the enzyme has been found to contain an average of 3.4 moles of zinc per mole of enzyme of molecular weight 1×10^6 (12) and that dissociation of the enzyme by DPNH or 1,10-phenanthroline yields 4 subunits (1). Not all the binding sites are catalytically active; specifically, the data show that for each DPN or DPNH molecule bound to an active site, a 2nd molecule may also bind, but to a noncatalytically active site. Thus only half the sites which bind DPN or DPNH are enzymatically active sites. This concept is supported by the finding of van Eys *et al.* (13) that for a number of dehydrogenases, 2 molecules of DPN are bound per mole of substrate analogue. It is interesting to note that the kinetic and sedimentation data indicate that only half the binding sites for DPN or DPNH are available for TPN or TPNH; specifically those binding sites which are enzymatically active. It is always possible, of course, that TPN or TPNH may bind to nonactive sites but that such binding would have no effect on the kinetic or sedimentation behavior. In view of the large effects caused by DPN or DPNH binding to nonactive sites, such a possibility seems extremely unlikely.

A general scheme for dehydrogenase action has been suggested by van Eys *et al.* (14) which requires the presence of 2 DPN molecules for enzymatic reaction to proceed. The conclusion of these workers is that a bound, nonactive, DPN molecule is necessary for the reaction to proceed. Derivations of kinetic equations for the mechanism proposed are complex, and it is not certain that the kinetic data would fit such equations. However, such a mechanism does not appear to hold for glutamic dehydrogenase on the basis of data so far presented. It is clear that the presence of 2 DPNH molecules causes inhibition of the reaction as well as dissociation of the enzyme. On the other hand, the presence of 2 DPN molecules causes activation and a further increase in sedimentation coefficient. The affinities of these two sites for DPN are so different that at concentrations of DPN approximately equal to the Michaelis constant, less than 4 per cent of the DPN would be bound to the activating site.

One of the puzzling aspects of this study is why DPNH causes dissociation of the molecule whereas DPN does not. Sedimentation experiments have been carried out with conditions under which both the active and activating sites are almost completely occupied by DPN, yet there is no decrease in the sedimentation coefficient. One possibility is that the difference between these coenzymes may result from differences in the manner by which they are bound to the enzyme. If this were true, the dissociation of the enzyme may be a valuable tool in determining the groups involved in coenzyme-binding. On the other hand, the difference between these coenzymes might result from their different charge which would influence the association-dissociation equilibrium. The nature of the forces involved in the association-dissociation reaction has not yet been investigated and should prove to be an interesting study. The unusual temperature effects on the Michaelis constant for DPN as contrasted to the Michaelis constant for DPNH undoubtedly result, in part, from the effect of temperature on the association-dissociation equilibrium.

SUMMARY

1. Crystalline beef liver glutamic dehydrogenase is a dissociable enzyme. The coenzymes for the reaction, diphosphopyridine nucleotide (DPN), its reduced form, triphosphopyridine nucleotide, and its reduced form, have been investigated for their effects upon both the association-dissociation reaction and the rate of the enzymatic reaction. The experiments were all made at pH 8.0 in tris(hydroxymethyl)aminomethane-acetate buffers.

2. The kinetic and sedimentation data show that the associa-

tion-dissociation behavior exhibited by this enzyme must be directly related to its catalytic function.

3. At coenzyme concentrations of less than 3×10^{-4} M, the presence of any of the coenzyme favors the association of enzyme subunits, as represented by an increase in the sedimentation coefficient of the enzyme. Reduced DPN differs from the others in that concentrations greater than 3.5×10^{-4} M cause dissociation of the enzyme as represented by the appearance of two distinct peaks in the sedimentation patterns. DPN differs from the triphosphopyridine nucleotides in that the sedimentation coefficient in the presence of DPN is slightly higher than those in the presence of either triphosphopyridine nucleotide or its reduced form.

4. Correlation of the sedimentation and kinetic data show that association of the enzyme results in increasing rates of the enzymatic reaction, whereas dissociation results in inhibition, the enzyme being inactive when dissociated.

5. It is found that all the coenzymes are bound to the same active site of the enzyme, but that in addition, DPN and its reduced form are bound to a second, noncatalytically active, site.

REFERENCES

1. Frieden, C., *Biochim. et Biophys. Acta*, **27**, 431 (1958).
2. Olson, J. A., and Anfinsen, C. B., *J. Biol. Chem.*, **197**, 67 (1952).
3. Strecker, H. J., *Arch. Biochem. Biophys.*, **41**, 128 (1953).
4. Olson, J. A., and Anfinsen, C. B., *J. Biol. Chem.*, **202**, 841 (1953).
5. Alberty, R. A., Massey, V., Frieden, C., and Fuhlbrigge, A. R., *J. Am. Chem. Soc.*, **76**, 2485 (1954).
6. Kistiakowsky, G. B., and Rosenberg, A. J., *J. Am. Chem. Soc.*, **74**, 5020 (1952).
7. Frieden, C., *J. Biol. Chem.*, **234**, 815 (1959).
8. Alberty, R. A., and Massey, V., *Biochim. et Biophys. Acta*, **13**, 347 (1954).
9. Hayes, J. E., Jr., and Velick, S. F., *J. Biol. Chem.*, **207**, 225 (1954).
10. Alberty, R. A., *J. Am. Chem. Soc.*, **80**, 1777 (1958).
11. Frieden, C., *J. Am. Chem. Soc.*, **79**, 1894 (1957).
12. Adelstein, S. J., and Vallee, B. L., *J. Biol. Chem.*, **233**, 589 (1958).
13. van Eys, J., Stolzenbach, F. E., Sherwood, L., and Kaplan, N. O., *Biochim. et Biophys. Acta*, **27**, 63 (1958).
14. van Eys, J., San Pietro, A., and Kaplan, N. O., *Science*, **127**, 1443 (1958).

Glutamic Dehydrogenase

II. THE EFFECT OF VARIOUS NUCLEOTIDES ON THE ASSOCIATION-DISSOCIATION AND KINETIC PROPERTIES*

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The previous paper has shown that for beef liver glutamic dehydrogenase, an enzyme which undergoes reversible dissociation, there is a direct correlation between the initial velocity and sedimentation behavior of the enzyme as a function of the coenzyme concentration (1). The kinetic studies carried out showed that the effects of the coenzyme on the initial velocity of the enzymatic reaction may be interpreted in terms of the association-dissociation behavior of the enzyme, a conclusion which should extend to the effects of other compounds on the reaction velocity. It is the purpose of the present investigation to study the effects of various compounds on the kinetic and molecular properties of the enzyme. It will be shown that compounds which have rather striking effects on the over-all reaction velocity exert these effects by influencing the degree of association or dissociation of the enzyme molecule. Furthermore, these effects are dependent on the coenzyme used, since the coenzymes themselves have different effects on the association-dissociation reaction. Most of the compounds studied bear some structural relation to portions of either the di- or triphosphopyridine nucleotide molecule, and those which exhibit the most unusual kinetic effects are the adenosine nucleotides. However, in addition, some experiments have been performed with 1,10(ortho)-phenanthroline, a compound that causes dissociation of the enzyme into four subunits (2). The results support the conclusions of the previous paper concerning the presence of active and nonactive binding sites on the enzyme molecule (1).

EXPERIMENTAL

The kinetic and sedimentation experiments were carried out as described in the previous paper (1). All kinetic experiments were conducted at pH 8.0 in 0.01 M Tris-acetate[1] buffer. When DPN or TPN was used as coenzyme, the concentration of L-glutamate used was 0.05 M. When DPNH or TPNH was used as coenzyme, the reaction mixture was 0.05 M with respect to α-ketoglutarate and 0.1 M with respect to NH_4Cl.

Reagents—Most of the nucleotides used were obtained from the Sigma Chemical Company, except that UDP, CDP, 5′-IMP and the deamino analogue of DPN were products of Pabst Laboratories, and 2′-AMP was obtained from Schwarz Laboratories. Reduced deamino DPN was prepared by chemical reduction with hydrosulfite. The oxidized and reduced *N*-methylnicotinamides were obtained from Dr. G. R. Drysdale. NMN was prepared from DPN with the use of snake venom pyrophosphatase as described by Kaplan and Stolzenbach (3). This NMN was obtained free of metal ions by means of a Dowex 50 column (Na^+ form). Reduced NMN was prepared from metal-free NMN by use of hydrosulfite. The NMNH was then precipitated as the barium salt from alcohol. For use in kinetic experiments, Ba^{++} was removed with excess Na_2SO_4. The α-ketoglutarate was recrystallized before use from acetone-benzene mixtures, and the sodium-L-glutamate used was over 99 per cent pure. 1,10-phenanthroline was a product of G. Frederick Smith Chemical Company. The crystalline beef liver glutamic dehydrogenase was obtained from C. F. Boehringer, Germany.

Methods—When varying concentrations of DPNH were used as coenzyme at a particular concentration of inhibitor, the results were plotted as the ratio of velocity with and without inhibitor as a function of coenzyme concentration. This procedure was adopted because of the inhibition by DPNH which would make interpretation of the usual Lineweaver-Burk plot (4) difficult. It may be shown that results plotted this way differ depending on whether the inhibition is competitive, noncompetitive, or uncompetitive and that the inhibition constants may be calculated from this type of plot. For TPNH or TPN as coenzyme, the usual Lineweaver-Burk plot may be employed. At 25°, the Michaelis constant and activation constant for DPN are different enough so that at low DPN concentrations, the usual Lineweaver-Burk plot may also be used.

RESULTS

The effect of coenzyme on the sedimentation behavior and kinetic properties of glutamic dehydrogenase have been described in the previous paper (1). The experiments reported in the present paper represent attempts to find compounds which are bound to the same binding sites as the coenzymes and thereby influence both the kinetic properties and the association-dissociation behavior of the enzyme.

Effects of Adenosine Nucleotides on Initial Velocity and Sedimentation Behavior of Enzyme—The previous paper has shown that neither TPN nor TPNH is bound to a nonactive site of the enzyme even though the active site for all the coenzymes is the same (1). Thus, it appeared likely that compounds which resembled the adenosine nucleotide moiety of the diphosphopyridine nucleotides might be bound to the nonactive site also. Adenosine, 2′- and 5′-AMP, ADP, and ATP have been thoroughly investigated with respect to their influence on the initial

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[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

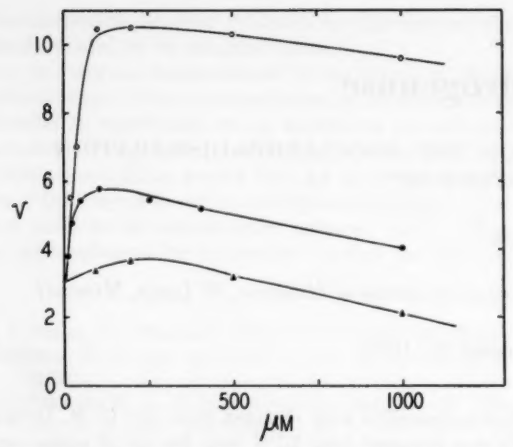

FIG. 1. The effect of increasing concentration of adenosine (△), 5′-AMP (●) and ADP (○) on the enzymatic oxidation of TPNH. Experiments conducted at 25°, pH 8.0, in 0.01 M Tris-acetate at a TPNH concentration of 1.2×10^{-4} M. α-Ketoglutarate and NH_4Cl concentrations were 0.05 and 0.1 M, respectively.

velocity of the enzymatic reaction and the sedimentation behavior of the enzyme. Since experiments involving TPN or TPNH should be most readily interpretable, these results are presented first.

TPNH—Fig. 1 shows the effect of increasing concentrations of adenosine, 5′-AMP, and ADP on the initial velocity of the enzymatic reaction at constant TPNH concentration. This figure shows that these three compounds increase the rate of TPNH oxidation and that the activation by ADP is greater than that by the 5′-AMP which in turn is greater than that by the adenosine. The extent of activation by these compounds is essentially independent of the TPNH concentration, indicating that TPNH does not compete for the site to which the AMP or ADP is bound. As the concentration of adenosine, 5′-AMP or ADP is increased, however, it competes with TPNH for the active site of the enzyme as demonstrated by inhibition of the reaction (Fig. 1).

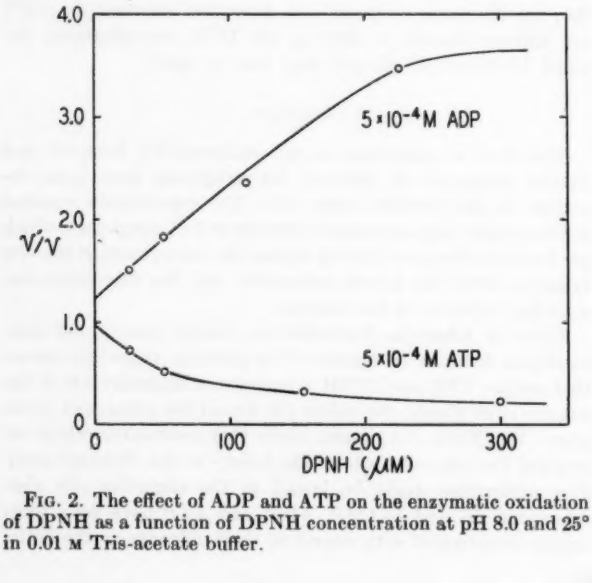

FIG. 2. The effect of ADP and ATP on the enzymatic oxidation of DPNH as a function of DPNH concentration at pH 8.0 and 25° in 0.01 M Tris-acetate buffer.

At constant TPNH concentration inhibition by adenosine is greatest, and it may be calculated that the inhibition constant for adenosine is at least 4×10^{-4} M. On the other hand, the activation constant for 5′-AMP or ADP is about 4×10^{-5} M. The inhibition constants for 5′-AMP and ADP are larger than 4×10^{-4} M, and it is clear that the affinities of the two sites, active and nonactive, for the adenosine nucleotides are markedly different. As distinct from the 5′-AMP and ADP, the 2′-AMP shows no activation of the reaction at any concentration. This compound is only inhibitory, and the inhibition constant is about 5×10^{-3} M when either TPNH or DPNH is used.

Whereas the 5′-AMP and ADP increase the initial velocity, ATP shows practically no effect on the enzymatic rate when TPNH is the coenzyme. Experiments with ATP and TPNH yielded rather erratic results, sometimes showing slight activation and sometimes slight inhibition, but the rate in the presence of ATP, up to 2×10^{-2} never differed by more than 15 to 20 per cent from the rate in its absence. However, it may be shown that ADP and ATP are bound to the same site of the enzyme since 5×10^{-3} M ATP, although having almost no effect by itself on the reaction velocity, will almost completely remove the activating effect of 5×10^{-4} M ADP.

TPN—Initial velocity studies with TPN used as coenzyme are similar to those in which TPNH is used except that in addition to 5′-AMP and ADP, the presence of ATP will also activate the enzymatic reaction. As with TPNH, the activation by these compounds is essentially independent of the TPN concentration.

DPNH—The fact that both TPN and TPNH are bound only to the catalytically active site of the enzyme makes interpretation of the above results quite straightforward in terms of simple activation and inhibition. The data obtained with DPNH, however, are remarkably different as a consequence of the fact that DPNH binding at both the active and nonactive sites results in dissociation of the enzyme to its inactive form. Thus compounds which affect the binding of DPNH at either one of these sites may have a great influence on the reaction velocity.

Fig. 2 shows the effect of ADP and ATP on the initial velocity as a function of DPNH concentration. These data are plotted as the ratio of initial velocity in the presence of 5×10^{-4} M ADP or ATP to the velocity in the absence of ADP or ATP (v':v). Fig. 2 shows that v':v *increases* with increasing DPNH concentration at constant ADP concentration but *decreases* with increasing DPNH concentration at constant ATP concentration. In this particular experiment, there is a difference of 14-fold in the initial velocity at a DPNH concentration of 3×10^{-4} M.

In order to show that ADP and ATP are affecting the kinetic behavior by influencing the extent of dissociation of the enzyme, sedimentation velocity experiments have been conducted in the presence of DPNH with and without ADP and with and without ATP. The results of these experiments are shown in Fig. 3 and were made at a protein concentration of 3.2 mg. per ml. In Fig. 3 *A*, the concentration of DPNH in both the lower and upper cell is 3.8×10^{-4} M. In addition, the solution in the upper cell is 5×10^{-4} M with respect to ATP. At this particular DPNH concentration, about 10 per cent of the protein is dissociated as represented by the area in the lower cell. The sedimentation coefficients of the two peaks are 24.5 and 15 S. The addition of ATP, the upper cell in this figure, shows that the effect of DPNH has been enhanced and dissociation is almost complete. The peak in the presence of ATP and DPNH has

a sedimentation coefficient of 13.7 S and is only slightly skewed. At the same concentration, ATP in the absence of DPNH has no effect on the sedimentation velocity behavior. Fig. 3 *B* shows the effect of ADP (upper cell) on dissociation by DPNH. The concentration of DPNH in both cells is 4.6×10^{-4} M, which is high enough to yield an area of about 40 to 50 per cent for the slow moving peak. The sedimentation coefficients of the two peaks in the lower cell are 21.0 and 12.3 S. To the upper cell, 2×10^{-4} M ADP has been added, and the dissociation of the enzyme by DPNH is almost completely reversed. The sedimentation coefficient for the peak in the upper cell is 26.9 S, and the peak is slightly skewed. ADP in the absence of DPNH increases the sedimentation coefficient of the enzyme, but not to a very great extent.

Adenosine and 5′-AMP act in a manner similar to ADP, although the effects of adenosine are not as striking as for the AMP or ADP. That these compounds are binding at the same site as ADP is shown by the fact that at an adenosine or 5′-AMP concentration of 2×10^{-4} M, dissociation of enzyme by DPNH is almost completely reversed, the results being almost identical to those shown in Fig. 3 *B*.

It was shown above that ADP and ATP compete for the same site of the enzyme molecule in the oxidation of TPNH. Similar results are obtained for the oxidation of DPNH as well. In addition, the inhibition constant of ATP (uncompetitive inhibition) is 3.5×10^{-5} M, almost identical with the activation constants for the 5′-AMP and ADP. Thus there is little doubt that the same site is involved in the binding of 5′-AMP, ADP, and ATP.

DPN—Since DPN does not cause dissociation of the enzyme at high concentrations, the influence of the adenosine nucleotides will quite naturally be different from that for DPNH. Typical results, plotted as reciprocal initial velocity against reciprocal DPN concentrations, in the presence and absence of 5×10^{-4} M ADP are shown in Fig. 4. These data were obtained at 25°, pH 8.0, in 0.01 M Tris-acetate buffer. In the absence of ADP, the Michaelis and activation constants for DPN are calculated to be 0.7×10^{-4} M and 2×10^{-3} M, respectively. However, in the presence of ADP, the plot is linear with a Michaelis constant of 0.9×10^{-4} M. A comparison of the affinity of the activating site for DPN and ADP shows that at the concentration of ADP used, essentially no DPN is bound to the activating site even at the highest DPN concentrations used in this experiment. Not only does the ADP prevent DPN binding, but the presence of the nucleotide activates the enzymatic reaction similar to its activation of TPNH oxidation, although it would appear from Fig. 4 that DPN is a better activator of the reaction than is ADP. This plot also shows that the activation of the reaction by a particular concentration of ADP will become progressively less, the higher the DPN concentration. The 5′-AMP and ATP act in a similar manner to ADP, although ATP does not seem to activate the reaction as well as 5′-AMP or ADP. The formation of an enzyme-DPN-ATP complex does not cause dissociation of the enzyme as observed by sedimentation experiments.

Results with DPN, DPNH, TPN, and TPNH, which illustrate their behavior with ADP and ATP are summarized in Table I. Data are given as the ratio of the velocities in the presence and absence of ADP or ATP. The table shows the effect of the adenosine nucleotide at high and low concentration of coenzyme. Adenosine and 5′-AMP are not listed since their effects are similar to ADP, and different only in magnitude.

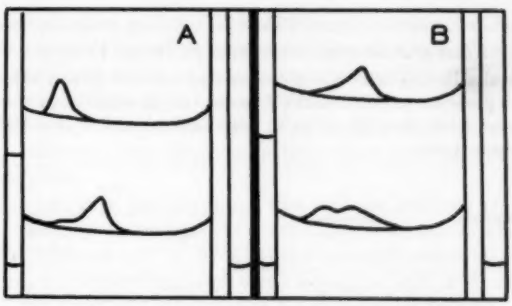

FIG. 3. Sedimentation diagrams illustrating the effect of (*A*) ATP and (*B*) ADP on the dissociation of enzyme by DPNH. In both experiments, two double sector centrifuge cells were used; the lower pattern shows the effect of DPNH alone and while the upper pattern is the result of addition of either (*A*) ATP or (*B*) ADP in the presence of DPNH. Pictures taken at 40 minutes after reaching a speed of 42,040 r.p.m. Average rotor temperature was 9° and 4° for (*A*) and (*B*), respectively, and the direction of sedimentation is from left to right. Protein and nucleotide concentrations are given in text.

Effects of Other Nucleotides on Enzymatic Reaction—In order to determine whether the effects so far described are specific for the adenine moiety, several other nucleotides were tested for their effect on the oxidation of TPNH. If these compounds were bound at the same site as ADP, they would undoubtedly activate the reaction. However, 1×10^{-3} M concentrations of 5′-IMP, 5′-GMP, and UDP had only inhibitory effects on the reaction rate. CDP did not inhibit the reaction at this or at lower concentrations, but showed very slight activation (about 5 to 10 per cent). Thus, it would appear that the adenine moiety is essential for the binding of nucleotides to the nonactive site of the enzyme. In addition to the above nucleotides, the deamino analogues of DPN and DPNH have been tested. It has been observed previously that the deamino DPN showed no such activating effects as does DPN (5). This has been confirmed, and in addition it has been found that the deamino DPNH does not appear to show any inhibitory effects. The Michaelis con-

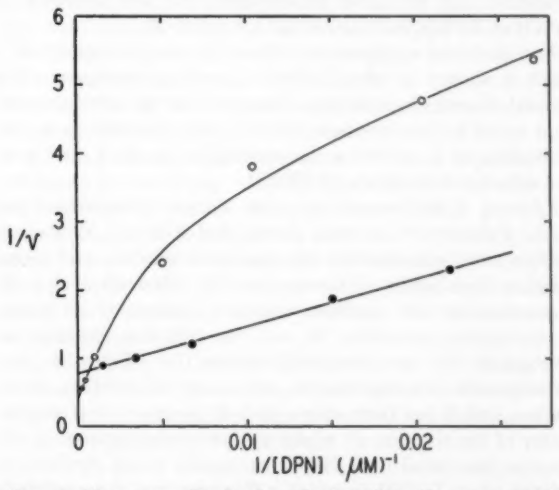

FIG. 4. Lineweaver-Burk plots for DPN in the presence (●) and absence (○) of 5×10^{-4} M ADP. Results obtained at 25°, pH 8.0, in 0.01 M Tris-acetate buffer at a glutamate concentration of 0.05 M.

TABLE I

Effect of Adenosine Nucleotides on Initial Velocity

Results in this table are given as the ratio of initial velocity in the presence of ADP or ATP to the initial velocity in the absence of ADP or ATP (v':v) at high and low DPN and DPNH concentrations.

Coenzyme	Concentration	v':v	
		ADP 5×10^{-4} M	ATP 5×10^{-4} M
DPN	5×10^{-5} M	2.2*	1.5
	1×10^{-3} M	1.3	1.2
DPNH	3×10^{-5} M	1.5	0.65†
	4×10^{-4} M	3.5‡	0.2†
TPN	All concentrations	3.0	2.8
TPNH	All concentrations	3.2	1.0

* Increases sedimentation coefficient over DPN alone at this DPN concentration.

† Enhances dissociation of the enzyme in the presence of DPNH.

‡ Prevents dissociation of the enzyme in the presence of DPNH.

stant for deamino DPNH was found to be about 0.8×10^{-4} M, and concentrations up to 1×10^{-3} M give no dissociation of the enzyme as observed in sedimentation experiments.

Effect of Nicotinamide Derivatives on Enzymatic Reaction—Nicotinamide, oxidized and reduced *N*-methylnicotinamide, NMN, and NMNH were all investigated for their effects on the initial rate of oxidation with TPNH and DPNH. None of these compounds exhibits any striking effects on the reaction velocity up to concentrations of about 5×10^{-4} M. Above these levels, the reduced compounds absorb too strongly to measure initial velocities. NMN shows a very slight tendency to activate the reaction up to concentrations of 1.5×10^{-3} M, but the activation is only about 25 per cent greater than in the absence of NMN. At levels of NMNH of about 5×10^{-4} M, there is only slight inhibition. In all these experiments, the concentration of TPNH or DPNH used was about 1.2×10^{-4} M.

The inhibition constant for oxidized *N*-methylnicotinamide is about 1×10^{-2} M when DPNH is used as coenzyme. The reduced *N*-methylnicotinamide, however, has an inhibition constant which is lower by about 10-fold. Nicotinamide up to concentrations of 2×10^{-2} M has essentially no effect on the initial velocity of oxidation of DPNH.

Effect of 1,10-Phenanthroline on Enzyme Dissociation and Initial Velocity—It has been shown that 0.02 M 1,10-phenanthroline completely inhibits the enzymatic reaction and causes complete dissociation of the enzyme (2). The effect of 1,10-phenanthroline was examined originally because of its known metal-chelating properties (6), and the fact that glutamic dehydrogenase is a zinc-containing enzyme (7). Thyroxine, also a strong metal-binding reagent, will cause dissociation of the enzyme, and it has been shown that thyroxine is a strong inhibitor of the reaction at relatively low concentrations (8, 9). Enzyme dissociated by 1,10-phenanthroline is not further dissociated when DPNH is added. However, the dissociation is prevented by the presence of coenzymes other than DPNH, although TPN and TPNH seem to be less effective than DPN. Such experiments demonstrate that coenzyme affects the binding of 1,10-phenanthroline, presumably by competing for the same binding site of the enzyme.

Experiments on the inhibition of the enzymatic reaction in the presence of 1,10-phenanthroline show that this inhibition is reversible under conditions similar to those used in the sedimentation experiments. Thus for an enzyme solution at pH 8.0 in 0.1 M Tris-acetate buffer at about 5–10°, and in the presence of 0.02 M 1,10-phenanthroline, there is little or no change in the initial velocity after dilution for assay up to periods of 1.5 hours. Furthermore, the ratio of initial velocities at different enzyme dilutions did not change over the same time period, as contrasted with results recently obtained for yeast alcohol dehydrogenase (10). In the kinetic experiments enzyme was normally added last to the cuvette containing the reaction mixture.

At low 1,10-phenanthroline concentrations ($< 2 \times 10^{-3}$ M) with any coenzyme, inhibition by 1,10-phenanthroline may be completely removed by the addition of ADP. For example, the velocity in the presence of 0.5×10^{-4} M ADP is almost the same with or without 1,10-phenanthroline even though the 1,10-phenanthroline alone (2×10^{-3} M) may cause up to 60 per cent inhibition of the velocity. These experiments show that 1,10-phenanthroline and ADP appear to compete for the same site.

DISCUSSION

A considerable number of different effects have been observed on the rate of the enzymatic reaction in the presence of adenosine nucleotides with the different coenzymes of glutamic dehydrogenase. Although results with TPN and TPNH are relatively simple to interpret, it is difficult to envisage a general scheme to encompass all the effects with the different coenzymes unless the results are interpreted in terms of the association-dissociation behavior of the enzyme and the specificity of its binding sites.

It is clear from the results of this paper that the nonactive site to which DPN and DPNH may be bound (1) is identical to the site to which the adenosine nucleotides are bound, and that the unusual effects of the adenosine nucleotides on the rate of the enzymatic reaction result from the fact that these nucleotides compete with DPN or DPNH for this nonactive site. ADP increases the initial velocity of DPNH oxidation, because it displaces the second inhibitory molecule of DPNH, thereby preventing the dissociation of the enzyme by DPNH to its inactive form. As a result, stimulation by ADP becomes greater at higher concentrations, up to the point at which the DPNH concentration is so high that it displaces the ADP. Increasing the DPNH concentration then causes a decrease in the rate of the enzymatic reaction. For example, in an experiment in which the concentration of ADP was only 5×10^{-5} M, v':v (the ratio of the velocity in the presence and absence of ADP) was 1.9 at a DPNH concentration of 2×10^{-4} M, but had decreased to 1.3 at a DPNH concentration of 5×10^{-4} M. In direct contrast to ADP, ATP when bound to the nonactive site with DPNH as coenzyme, enhances the dissociation by DPNH. Since the dissociated enzyme is inactive, the ratio v':v will decrease with increasing DPNH concentration when the concentration of ATP is held constant. As with ADP, this ratio will again begin to approach 1 when the DPNH concentration gets so high that it displaces the ATP. For this type of inhibition, where the inhibitor has no effect unless the DPNH

is bound, the maximal velocity and Michaelis constant are decreased by the same factor, namely by $(1 + I/K_I)$.

Such results for DPNH are different from those obtained with TPNH as coenzyme, since TPNH cannot bind to the nonactive site of the enzyme. As a result, TPNH cannot cause dissociation of the enzyme nor can it displace ADP or ATP from the nonactive site no matter how high the concentration. Therefore, ADP activates the reaction, but the ratio $v':v$ is independent of TPNH concentration.

For DPN reduction, ADP causes activation similar to the activation of TPN or TPNH. However, since DPN and ADP may compete for the nonactive site, ADP can prevent DPN activation. However, high concentrations of DPN will displace ADP from the nonactive site so that the ratio $v':v$ will again depend on the DPN concentration.

All the effects of the various nucleotides on the rate of the enzymatic reaction with the different coenzymes may be qualitatively described in terms of the postulated active and nonactive site, the different specificities of these two sites, and the association-dissociation behavior of the enzyme. However, in the quantitative description of the effect of the adenosine nucleotides, the number of factors which must be considered is quite large as exemplified by the ADP activation of DPNH oxidation. Unfortunately the effects overlap to some extent, so that in any particular experiment, there is a composite of factors influencing the reaction. However, the derivation of equations which describe the experimental data on the basis of the proposed active and nonactive sites is straightforward. Furthermore, the data so obtained for one coenzyme are consistent with data for other coenzymes. For example, the binding constant of ADP is about 3.5×10^{-5} M regardless of whether it is determined from data obtained with DPN, DPNH, TPN, or TPNH.

It is quite apparent that the active and nonactive sites of the enzyme are different with respect to their affinity for different nucleotides. Thus the active site, in addition to binding either the di- or triphosphopyridine nucleotides, also binds adenosine, nicotinamide derivatives, and most other nucleotides, although some of these compounds are only poorly bound. The nonactive site in addition to adenosine, is specific only for the 5′-adenosine nucleotides as exemplified by AMP, ADP, ATP, DPN, and DPNH. Most other nucleotides tested, such as the 2′-AMP, 5′-IMP, 5′-GMP, UDP, TPN, TPNH, and the deamino analogue of either DPN or DPNH are not bound to this site. Such differences raise the possibility that the manner by which these compounds are bound, perhaps even the groups involved in the binding, may be different for the two different sites.

With respect to the specificity of the nonactive site, it is interesting to note some results recently obtained by van Eys *et al.* (11) on the activation of DPN reduction by nucleotides for yeast alcohol dehydrogenase. These workers found that either ADP or ATP caused an increase in initial velocity of DPN reduction, but that the activation was not specific for adenosine nucleotides, and the maximal effect occurred at rather high nucleotide concentrations.

The effects of ADP and ATP on isolated glutamic dehydrogenase are undoubtedly different from their effects *in vivo*. However, since glutamic dehydrogenase is present within the liver mitochondria in high concentrations, the unusual activator and inhibitory effects of these nucleotides on the initial velocity may very well need to be considered with respect to their role in oxidative phosphorylation. This is especially so because of the difference that these nucleotides show with the different coenzymes.

In discussing the results of this and the previous paper, it must be emphasized that the kinetic data obtained for glutamic dehydrogenase may be described in a straightforward manner in terms of the postulated active and nonactive sites of the enzyme and the specificity of such sites for different nucleotides. It is well known that there are many cases in which one cannot distinguish among two or more different mechanisms from kinetic data alone. However, for the glutamic dehydrogenase, the direct relation between molecular and kinetic properties allows the choice of some details of the reaction mechanism with a much greater degree of certainty. It must be remembered that the kinetic data for glutamic dehydrogenase may be very different from that obtained for other dehydrogenases because of the effects of the association-dissociation behavior on the catalytic properties of the enzyme. This difference, however, may be quite useful as a method for the clarification of specific details of the reaction mechanism of other dehydrogenases. It is clear that the details of a general scheme for dehydrogenase action must await further study on more dehydrogenases.

SUMMARY

1. Various compounds, most of which are structurally related to portions of the di- or triphosphopyridine nucleotide molecule, have been tested for their effects on the initial velocity of the beef liver glutamic dehydrogenase reaction when diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and the reduced form of each are used as coenzymes. The experiments were performed at pH 8.0, in 0.01 M tris(hydroxymethyl)aminomethane-acetate buffer at constant and noninhibitory levels of glutamate or α-ketoglutarate and NH_4^+.

2. It is found that some compounds, specifically the adenosine nucleotides, have a considerable effect on the reaction velocity and that such effects depend on which coenzyme is used.

3. The data are interpreted in terms of the postulated active and nonactive binding sites of the enzyme, the specificity of such sites, and the effect of the different coenzymes on the association-dissociation behavior of the enzyme.

4. In addition to DPN and its reduced form, only adenosine, adenosine 5′-phosphate, adenosine diphosphate, and adenosine triphosphate are tightly bound to the nonactive site. Compounds which are bound to this site either very poorly or not at all include TPN, its reduced form, adenosine 2′-phosphate, inosine 5′-phosphate, guanosine monophosphate, uridine diphosphate the deamino analogue of DPN or DPNH and several nicotinamide derivatives.

5. In addition to DPN, DPNH, TPN, and TPNH, the active site may bind all the compounds listed above. However, the affinity for these compounds is considerably less than the affinity for the coenzymes of the reaction.

REFERENCES

1. Frieden, C., *J. Biol. Chem.*, **234**, 809 (1959).
2. Frieden, C., *Biochim. et Biophys. Acta*, **27**, 431 (1958).
3. Kaplan, N. O., and Stolzenbach, F. E., in S. P. Colowick, and N. O. Kaplan, *Methods in enzymology, Vol. III*, Academic Press, Inc., New York, 1957, p. 899.

4. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
5. Olson, J. A., and Anfinsen, C. B., *J. Biol. Chem.*, **202**, 841 (1953).
6. Kolthoff, I. M., Leussing, D. L., and Lee, T. S., *J. Am. Chem. Soc.*, **73**, 390 (1951).
7. Vallee, B. L., Adelstein, S. J., and Olson, J. A., *J. Am. Chem. Soc.*, **77**, 5196 (1955).
8. Wolff, J., and Wolff, E. C., *Biochim. et Biophys. Acta*, **26**, 387 (1957).
9. Caughey, W. S., Smiley, J. D., and Hellerman, L., *J. Biol. Chem.*, **224**, 591 (1957).
10. Williams, R. J. P., Hoch, F. L., and Vallee, B. L., *J. Biol. Chem.*, **232**, 465 (1958).
11. van Eys, J., Ciotti, M. M., and Kaplan, N. O., *J. Biol. Chem.*, **231**, 571 (1958).

The Thyroxine-binding Protein of Bovine Synovial Fluid*

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The α- and β-globulin fractions of normal bovine synovial fluid have a higher proportion of hexosamine-containing glycoprotein[1] than the corresponding serum (1). Whether these fluid proteins have a specific function in synovial fluid, or ground substance generally, is as yet an open question. Some of the proteins may exist as complexes with hyaluronic acid (2–4); however, glycoproteins having biological activity in serum may serve the same purposes in extracellular fluids. Thus the thyroxine-binding protein appears to be present in lymph (5–8) suggesting that it complexes thyroxine in the extracellular fluid as it does in serum.

Electrophoretic evidence shows that the thyroxine-binding protein belongs to the class of α-globulins separated at pH 8.6 and the M-2 fraction separated at a pH of 4.5 (9–12). All available evidence leads to the conclusion that this protein has an acid isoelectric point and therefore may belong to the class of acid glycoproteins. However, since it has not been isolated, it is not possible to say with certainty that it is a hexosamine-containing glycoprotein. Some data suggest the probable presence of carbohydrate (13–15).

The object of this paper is to show the presence of thyroxine-binding protein in normal bovine synovial fluid. This is accomplished by determining the protein-bound iodine of synovial fluid, by demonstrating that added I^{131}-labeled thyroxine migrates electrophoretically in the α-globulin region, and by comparing the thyroxine-binding capacity of the α-globulin of fluid with that of serum.

EXPERIMENTAL

Synovial fluids and sera from heifers[2] were collected and handled as previously described (1). In all cases, studies were begun immediately to obviate any changes due to storage. Total proteins were determined by a biuret method (16) while protein-bound iodine was determined by the procedure of Meyer *et al.* (17). With the latter, direct precipitation of proteins from synovial fluid was hindered by the presence of hyaluronic acid. Precipitation was successful only after 2 ml. of fluid were first treated for 3 hours with 0.1 ml. of hyaluronidase solution (0.4 mg. of enzyme per ml. of saline) at 37°. Then zinc sulfate and sodium hydroxide were added in the prescribed manner (17).

For the electrophoresis experiments, 4 ml. of serum and approximately 4 μc. of I^{131}-labeled thyroxine (dissolved in 50 per cent propylene glycol)[3] were diluted to 5 ml. with water. With synovial fluid, 15 ml. were first treated with 0.75 ml. of the hyaluronidase solution and then concentrated by dialysis at 5° against a 20 per cent (by weight) solution of dextran.[4] This concentrate and 4 μc. of I^{131}-labeled thyroxine were combined and diluted to 5 ml. Then the mixtures were allowed to stand at room temperature for at least 30 minutes (12) before a 3-ml. aliquot was applied to the starch block for electrophoresis by the procedure previously described (1). The Veronal buffer had an ionic strength of 0.075 and a pH of 9.1. After electrophoresis for 20 hours at 5°, the starch block was cut into successive 1-cm. segments each of which was then eluted with 5 ml. of a 1 per cent solution of salt. The protein content of the eluates, was measured by the Lowry method (18). For measurements of radioactivity, 1-ml. aliquots were dried on aluminum planchets and counted with a Geiger-Müller counter.

RESULTS AND DISCUSSION

Protein-bound iodine levels were determined for a series of 15 cattle, Table I. Since it was necessary to pretreat the synovial fluid with hyaluronidase, a preliminary experiment was performed to determine any undesirable effect of the enzyme. The protein-bound iodine for 0.1 ml. of hyaluronidase solution and 0.9 ml. of bovine serum was the same as for 0.9 ml. of serum and 0.1 ml of a 1 per cent solution of sodium chloride. For bovine serum an average was obtained of 1.2 μg. of iodine per gm. of total protein, whereas for synovial fluid the average was 2.6 μg. of iodine per gm. Although the data for synovial fluid show considerable variability, on the average, the fluid value was about twice that of serum which is similar to the findings for canine lymph (5). The presence of appreciable protein-bound iodine does not prove the existence of the thyroxine-binding protein, however, as albumin alone could be the complex-forming protein.

To show the actual presence of thyroxine-binding protein, approximately 4 μc. of I^{131}-labeled thyroxine were added to serum or concentrated synovial fluid as already described. Fig. 1 compares the electrophoretic distribution of labeled thyroxine with the protein pattern for synovial fluid; serum gave qualitatively similar curves. In this figure one ordinate is the per cent of total protein in each segment of starch and the other the radioactivity in c.p.m. per ml. of eluate. The abscissa is the fraction, $R_{albumin}$, which is the distance migrated divided by the distance of the albumin peak from the origin. It is clear from Fig. 1 that the

* This project was supported by grants from the United States Public Health Service (A-523 (C8)) and the Michigan Chapter, Arthritis and Rheumatism Foundation.

[1] Glycoprotein is used in this paper in connection with any hexosamine-containing protein.

[2] Synovial fluids and sera were obtained through the courtesy of Standard Beef, Inc., Detroit, Michigan.

[3] Abbott Laboratories, Oak Ridge, Tennessee. Specific activity was 14 to 30 mc. per mg.

[4] Generous quantities of dextran were supplied by Commercial Solvents Corporation, Terre Haute, Indiana.

TABLE I

Protein-bound iodine of bovine serum and synovial fluid

Protein-bound iodine values are given in μg. of I per 100 ml. of fluid and total proteins are given in gm. per 100 ml.

Sample	Serum			Synovial fluid		
	Protein-bound iodine	Total protein	μg. I/gm. protein	Protein-bound iodine	Total protein	μg. I/gm. protein
57–3	4.7	8.2	0.6	1.6	1.3	1.2
57–5	10.2	7.0	1.5	4.5	1.8	2.5
57–12	10.2	5.7	1.8	3.6	1.6	2.3
57–13	7.3	7.3	1.0	1.5	0.9	1.6
57–15	12.8	6.8	1.9	5.9	0.7	8.4
57–16	12.4	6.2	2.0	6.5	0.8	8.1
57–17	13.8	7.2	1.9	7.5	1.5	5.0
58–1	7.6	6.9	1.1	1.6	0.8	2.0
58–3	7.0	6.8	1.0	2.7	1.6	1.7
58–6	8.5	5.9	1.4	3.2	1.0	3.2
58–7	11.2	8.0	1.4	5.3	2.0	2.6
58–8	5.2	7.7	0.7	1.3	1.6	0.8
58–9	5.5	7.7	0.7	0.0	0.7	0.0
58–11	2.5	7.9	0.3	0.0	0.9	0.0
58–13	4.5	7.7	0.6	0.2	1.1	0.2
Average	8.2	7.1	1.2	3.0	1.2	2.6
S.d.*	±3.4	±0.8	±0.5	±2.3	±0.4	±2.6

* Standard deviation.

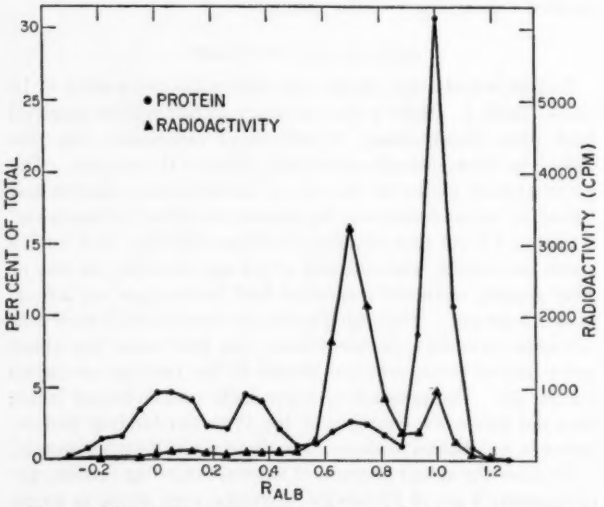

FIG. 1. Electrophoretic distribution of I^{131}-labeled thyroxine added *in vitro* to normal bovine synovial fluid.

added thyroxine migrated primarily with the α-globulin region thereby demonstrating the presence of the thyroxine-binding protein. Radiothyroxine added to the starch block in the absence of serum remained near the origin.

It was desirable to compare the amount of thyroxine binding by the α-globulin fraction of synovial fluid with that of serum. Two experiments were performed. First, before electrophoresis, the protein-bound iodine levels of serum and fluid samples were increased by adding varying amounts of unlabeled L-thyroxine

TABLE II

Effect of protein-bound iodine level on electrophoretic distribution of radiothyroxine

Samples separated electrophoretically consisted of 4 ml. of serum or concentrated synovial fluid (15 ml. of original fluid), unlabeled thyroxine and radiothyroxine adjusted to a final volume of 5 ml. Electrophoretic distribution of radioactivity is expressed in per cent while the protein-bound iodine level is in μg. I per gm. of protein.

Sample	Serum				Synovial fluid			
	Total protein-bound iodine	Radioactivity			Total protein-bound iodine	Radioactivity		
		α	Albumin	β and γ		α	Albumin	β and γ
1	2.0	93.2	4.7	2.1	5.1	78.3	9.2	12.5
3	3.9	75.9	14.8	9.3	3.8	76.6	14.6	8.8
6	6.8	78.0	14.7	7.3	6.8	72.2	16.8	11.0
13	16.6	54.2	20.6	25.2	31.1	45.4	21.6	33.0
9	62.5	32.5	28.9	38.6	114.3	33.7	34.3	32.0

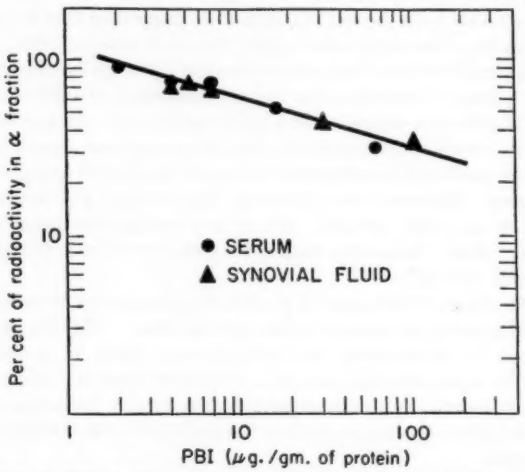

FIG. 2. Effect of increasing protein-bound iodine (PBI) on the distribution of radiothyroxine in the α-globulin fraction of bovine sera and synovial fluids.

(dissolved in 50 per cent propylene glycol) in addition to the radiothyroxine. For the preparation of these solutions, 0.0 to 24.2 μg. of unlabeled L-thyroxine and 0.17 to 0.67 μg. of radiothyroxine (approximately 4 μc.) were combined with 4 ml. of serum or concentrated synovial fluid (representing 15 ml. of original fluid). After diluting to 5 ml., a 3-ml. aliquot was used for the electrophoretic separation. The total protein-bound iodine in μg. per gm. of protein was also determined on suitable aliquots of the adjusted mixtures. The distribution of radioactivity in the albumin, α-, and the combined β- and γ-areas was evaluated planimetrically. The relative proportions, as well as the corresponding protein-bound iodine levels, are given in Table II. In Fig. 2 the proportion of radioactivity in the α-globulin region is plotted against total protein-bound iodine on log-log paper (10). Since all of the points are on a single straight line, it appears that the amount of thyroxine binding by α-globulin is the same for synovial fluid and serum.

In a second study, serum and synovial fluid samples, adjusted to protein-bound iodine levels between 6.8 and 114 μg. per gm. of protein in the manner already described, were separated electrophoretically. Then, after electrophoretic separations, equal portions of the eluates, corresponding to the α-globulin region, were combined and aliquots of these mixtures were used for determinations of total protein (18) and iodine. For the latter, 10-ml. samples were dried and ashed with sodium carbonate without prior precipitation of the protein. No interference in the iodine determination was observed from the presence of the barbital buffer used for electrophoresis. In three samples of serum and synovial fluid the concentration of iodine was between 1.4 and 2.2 μg. of iodine per 100 mg. of α-globulin protein regardless of the original protein-bound iodine level. Thus the thyroxine-binding protein had been saturated with thyroxine. There were no significant differences between the iodine contents of the α-globulin regions of serum and synovial fluid. This confirmed the conclusion that the thyroxine-binding capacity was the same for both tissues.

SUMMARY

The protein-bound iodine for normal bovine synovial fluid was found to be about twice that of serum; 2.6 μg. of iodine per gm. of total protein for fluid compared with 1.2 μg. of iodine per gm. for serum.

Electrophoresis on starch of serum and synovial fluid, containing I^{131}-labeled thyroxine, showed the presence of the thyroxine-binding protein in both. Experiments correlating the relative proportions of radiothyroxine in the α-globulin regions with increasing protein-bound iodine levels showed that the thyroxine-binding capacities of synovial fluid is the same as serum. This was confirmed by the observation that the α-globulin region of synovial fluid and serum, containing excess added thyroxine, had the same concentrations of total iodine.

REFERENCES

1. Neuhaus, O. W., and Letzring, M., *J. Biol. Chem.*, **232**, 177 (1958).
2. Curtain, C. C., *Biochem., J.*, **61**, 688 (1955).
3. Ogston, A. G., and Sherman, T. F., *Nature*, **181**, 482 (1958).
4. Pigman, W., Holley, H. L., Platt, D., and Gramling, E., *Federation Proc.*, **17**, 289 (1958).
5. Salter, W. T., White, W. F., and McKay, E. A., *Federation Proc.*, **5**, 90 (1946).
6. Salter, W. T., *Transactions of the American Association for the Study of Goiter*, Charles C Thomas, Springfield, Illinois, 1942–1946, p. 145.
7. Salter, W. T., and Johnston, M. W., *J. Clin. Endocrinol.* **8**, 911 (1948).
8. Klitgaard, H. M., Toth, J. P., Kot, P. A. and Whaley, R. A., *Proc. Soc. Exptl. Biol. Med.*, **96**, 122 (1957).
9. Barker, L. B., in *The thyroid, Brookhaven symposia in biology No. 7*, Office of Technical Services, Department of Commerce, Washington, 1955, p. 74.
10. Robbins, J., and Rall, J. E., *J. Clin. Invest.*, **34**, 1324 (1955).
11. Albright, E. C., Larson, F. C., and Deiss, W. P., *J. Clin. Invest.*, **34**, 44 (1955).
12. Freinkel, N., Dowling, J. T., and Ingbar, S. H., *J. Clin. Invest.*, **34**, 1698 (1955).
13. Ingbar, S. H., Dowling, J. T., and Freinkel, N., *Endocrinology*, **61**, 321 (1957).
14. Boas, N. F., and Foley, J. B., *Endocrinology*, **56**, 474 (1955).
15. Schmid, K., *J. Am. Chem. Soc.*, **75**, 60 (1953).
16. Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F. *Am. J. Clin. Path.*, **18**, 723 (1948).
17. Meyer, K. R., Dickenman, R. C., White, E. G., and Zak, B., *Am. J. Clin. Path.*, **25**, 1160 (1955).
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).

The Inhibition of Beef Liver Glutamic Dehydrogenase by Metal-binding Agents*

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Crystalline preparations of the glutamic dehydrogenase of beef liver contain from 2 to 4 gm. atoms of zinc per mole of enzyme protein (1). The dependence of the catalytic activity of glutamic dehydrogenase on the metal is inferred from (*a*) the concomitant increase of zinc to protein and activity to protein ratios during its crystallization and (*b*) the inhibition of enzyme activity by agents which form stable complexes with zinc ions in aqueous solutions.

In this report the inhibition of glutamic dehydrogenase by metal-binding agents, including 1,10-phenanthroline, is shown to depend on the ability of these agents to form a complex with zinc ions and to be qualitatively similar to that observed with yeast alcohol dehydrogenase, another zinc metallodehydrogenase. A mechanism for the inhibition of glutamic dehydrogenase by metal-binding agents similar to that observed for another zinc metallodehydrogenase, yeast alcohol dehydrogenase, (2, 3) is proposed, although glutamic dehydrogenase differs from alcohol dehydrogenase both with respect to the reaction it catalyzes and its protein size and structure.

EXPERIMENTAL

The reagents employed in the enzymatic assay were analyzed by emission spectroscopy and found to contain no significant quantities of metals (see below) likely to interfere with metal-binding agents. The phosphate buffer employed has been shown to be free of metals except for calcium in very small amounts (1). Since calcium does not form complexes with most of the metal-binding agents employed in this study, its presence could be disregarded. All reagents were neutralized to pH 7.6 before use or analysis.

L(+)-Glutamic Acid—This was obtained from Eastman Organic Chemicals Department, Eastman Kodak Company. By emission spectroscopy (4, 5) the 1.1×10^{-2} M potassium glutamate contained the following metals (in μg. per ml.): Sr 0.01, Ba 0.01, Cr 0.3, Al 0.1, Mg 0.08, and a trace of calcium; Cd, Co, Fe, Mn, Na, Ni, P, Pb, and Zn were not detected.

DPN—A Pabst product (Lot No. 308). This was 95 to 99 per cent pure as estimated by cyanide complex formation (6); 8.3×10^{-3} M DPN contained the following metals (in μg. per ml.) Ba 0.37, Sr 0.05, Ca 15.0, and Mg 0.47. Al, Cd, Cr, Co, Fe, Mn, Ni, Pb, and Zn were not detected.

Metal-binding Agents—OP[1] (G. Frederick Smith Chemical Company); 8-hydroxyquinoline, sodium diethyldithiocarbamate, cupferron, pentanedione (Eastman); 2-carboxy-1-hydroxy-5-sulformazyl benzene (Zincon, La Motte Chemical Company); and EDTA disodium (Bersworth Chemical Company) were used without further purification.

Glutamic Dehydrogenase—This was prepared fresh from beef liver by the method of Olson and Anfinsen (7) and was recrystallized three times or was recrystallized from a preparation obtained from a commercial source (C. F. Boehringer and Soehne, Mannheim, Germany). The turnover numbers varied from 2400 to 3700 moles of DPN per minute per 10^6 gm.

The metal-free water was obtained and the buffers were prepared as described previously (1).

Enzymatic Assay—Enzyme activity was determined by following the change in optical density of DPNH at 340 mμ with a Beckman DU spectrophotometer. For the measurement of the rate of formation of DPNH the following 3.0-ml. reaction mixture was used at 23°: glutamate, 1.1×10^{-2} M; DPN, 8.3×10^{-3} M; 0.08 M potassium phosphate buffer at pH 7.8; and glutamic dehydrogenase, 3 μg. The enzyme was added last to start the reaction which was followed for 5 minutes. These concentrations provide optimal activity and a linear relation between enzyme concentration and velocity for optical absorbance changes of <0.100 per 5 minutes. The initial velocity of the reaction, V, was measured as change in optical absorbance per cm. per 5 minutes per 3 μg. of glutamic dehydrogenase, and was constant over this time interval; initial velocity was therefore measured. Relative activity is expressed as a fraction of the uninhibited control reaction, V_i/V_c.

In studies of the effects of metal-binding agents on the activity of other dehydrogenases, the time of contact between the agent and enzyme has been found to be a significant variable (2, 3, 8). When metal-binding agents are added to the reaction mixture with substrate and coenzyme before the addition of enzyme an *instantaneous*[2] inhibition is observed.

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[1] The abbreviations used are: OP, 1,10-phenanthroline; EDTA, ethylenediaminetetraacetate.

[2] "Instantaneous" is defined as the shortest time in which the reaction rate could be measured after all the compounds of the glutamic dehydrogenase reaction have been brought into contact.

When metal-binding agents are preincubated with the enzyme before assay, a *time-dependent* inhibition occurs. Therefore, two methods of testing the interaction were employed in which the duration of contact between agent and enzyme differed. (*a*) The enzyme was added last to a reaction cuvette containing the metal-binding agent, substrate, coenzyme, and buffer at 23°. The longest time of contact between enzyme and reagents before the velocity was measured was <5 seconds. This procedure is designated as *direct addition* and measures the *instantaneous* effect of the metal-binding agent. (*b*) The enzyme was first exposed to the metal-binding agent for a specified time under stated conditions of pH and temperature. These were then added jointly to a reaction cuvette containing substrate, coenzyme, and buffer. In this instance, the final concentration of metal-binding agent in the cuvette was $\frac{1}{15}$ of that initially in contact with the enzyme. The enzyme concentration in the preincubation mixture was 30 μg. per ml. This procedure is designated as *preincubation* and measures the *time-dependent* effect of the metal-binding agent.

RESULTS

Instantaneous Inhibition

Influence of Metal-binding Agents on Enzymatic Activity—The direct addition of sodium sulfide (1×10^{-2} M), sodium azide (3×10^{-1} M), OP (3×10^{-3} M), thiourea (3×10^{-1} M), diethyldithiocarbamate (3×10^{-2} M), 8-hydroxyquinoline (3×10^{-3} M), cupferron (3×10^{-3} M), EDTA (1.7×10^{-1} M), Zincon (6×10^{-5} M), and thyroxine (8×10^{-4} M) to the reaction cuvette all reduce the enzymatic activity of glutamic dehydrogenase (1). The concentrations given in parentheses are those which result in approximately 50 per cent inhibition. The relative activity as a function of the log of the concentration of the inhibitor is shown in Fig. 1 for three of the reagents, Zincon, 8-hydroxyquinoline, and EDTA, over the range in which they could be studied.

The instantaneous inhibition of glutamic dehydrogenase by OP is completely reversible on dilution. The degree of inhibition is a monotonically increasing function of the inhibitor concentration. In an assay mixture containing 0.8×10^{-3} M OP the enzyme exhibits 24 per cent of the controlled activity ($V_i/V_c = 0.24$). When this mixture is diluted with a solution containing all components of the system, except the inhibitor and enzyme, V_i/V_c increases. The enzyme shows 89 per cent of its full activity when the concentration of OP has been diluted to 2.67×10^{-3} M. When the OP concentration is 1.33×10^{-3} M or 1.33 M, V_i/V_c becomes 1.0. These percentages are exactly the same as those which are obtained when 2.67×10^{-3} M or 1.33×10^{-3} M OP are added to the assay system. Thus the reaction between glutamic dehydrogenase and OP causing instantaneous inhibition appears to reach a rapid and freely reversible equilibrium. This is equally true of the inhibition by 8-hydroxyquinoline and EDTA, but not with diethyldithiocarbamate.

Prevention of OP Inhibition by Metal Ions—It has been shown previously that the instantaneous inhibition of glutamic dehydrogenase is *reversed* by the addition of zinc chloride (1). In addition, metal ions *prevent* the occurrence of the instantaneous inhibition by OP. The effects of the divalent cations of the first transition series and of magnesium on the degree of glutamic dehydrogenase inhibition by OP are shown in Table I. Zinc ions have previously been shown to inhibit the enzyme (9). With the exception of magnesium, all these ions form strong complexes with OP and partially prevent the inhibition of the enzyme by the agent. Copper and iron, which form the strongest complexes with OP, prevent inhibition maximally. Zinc ions, which form strong complexes with OP, also prevent the inhibition of the enzyme by the reagent. Magnesium ions, which have little affinity for OP, do not prevent the inhibition at all.

The effects of varying added zinc concentration on the OP inhibition of glutamic dehydrogenase is presented in Fig. 2. A reduction of 45 per cent in relative activity (V_i/V_c) is brought about through the addition of 6.7×10^{-3} OP. Increasing concentrations of zinc chloride added to OP before the addition of enzyme to the reaction mixture progressively prevent inactivation. OP does not inhibit at all when the ratio of $[Zn^{++}]$:[OP] is 1 to 3. Addition of zinc chloride beyond this ratio again results in inhibition, this time due to the inhibiting effect of the zinc ion (9). Inactivation of the enzyme is complete when the ratio of $[Zn^{++}]$:[OP] becomes 1 to 1 (6.7×10^{-3} M $ZnCl_2$). The stoichiometry of the interaction of Zn^{++} and OP was also approached in the reverse direction, *i.e.* adding varying concentrations of OP to one inhibiting concentration of zinc ions. As

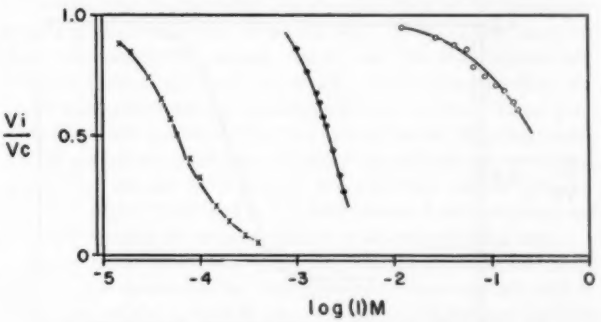

FIG. 1. Inhibition of liver glutamic dehydrogenase by Zincon, ×——×; 8-hydroxyquinoline, ●——●; and EDTA, ○——○. Direct addition; DPN → DPNH. Relative activity (V_i/V_c) as a function of log inhibitor concentration.

TABLE I

Prevention of instantaneous 1,10-phenanthroline (OP) inhibition

OP and metal ions were present in the reaction cuvette on addition of the enzyme. The initial reaction velocity, V, the relative activity, V_i/V_c, and the per cent restoration of activity are given.

	V	V_i/V_c	Recovery
			%
Control	0.102		
5.33×10^{-3} M OP	0.041	0.40	
5.33×10^{-3} M OP + 2×10^{-3} M Cu^{++} (blue)	0.090	0.88	80
5.33×10^{-3} M OP + 2×10^{-3} M Cd^{++}	0.079	0.77	62
5.33×10^{-3} M OP + 2×10^{-3} M Fe^{++} (red)	0.088	0.86	77
5.33×10^{-3} M OP + 2×10^{-3} M Co^{++} (pink)	0.073	0.72	52
5.33×10^{-3} M OP + 2×10^{-3} M Ni^{++} (blue)	0.066	0.65	35
5.33×10^{-3} M OP + 2×10^{-3} M Mn^{++}	0.078	0.76	61
5.33×10^{-3} M OP + 2×10^{-3} M Mg^{++}	0.042	0.41	2

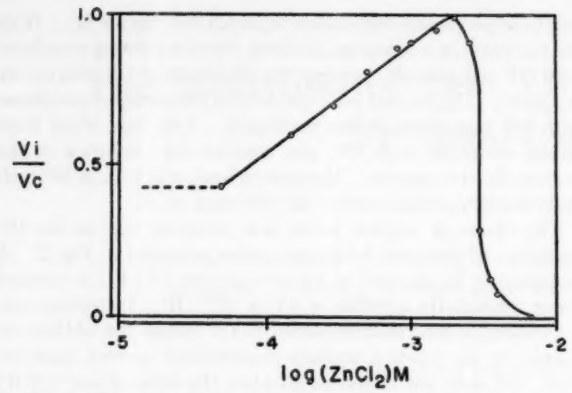

FIG. 2. The effect of varying zinc concentrations on the inhibition of glutamic dehydrogenase by 1,10-phenanthroline (OP). Relative activity (V_i/V_c) as a function of log ($ZnCl_2$) at a constant concentration of 6.7×10^{-3} M OP in the reaction cuvette. Direct addition; DPN → DPNH. Maximal restoration of activity at 2.2×10^{-3} M $ZnCl_2$ or a [Zn^{++}] to [OP] ratio of 1 to 3. Complete inactivation at 6.7×10^{-3} M $ZnCl_2$ or a [Zn^{++}] to [OP] ratio of 1 to 1. Potassium phosphate buffer, pH 7.8.

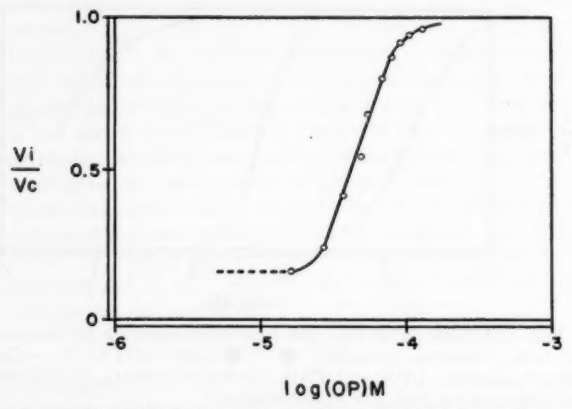

FIG. 3. The effect of varying 1,10-phenanthroline (OP) concentration on the inhibition of glutamic dehydrogenase by zinc ions. Relative activity (V_i/V_c) as a function of log (OP) at a constant concentration of 2.7×10^{-5} M $ZnCl_2$ in the reaction cuvette. Direct addition; DPN → DPNH; potassium phosphate buffer, pH 7.8. Restoration of activity begins at 2.2×10^{-5} M OP or a Zn to OP ratio of 1 to 1. Optimal restoration is achieved at 1.1×10^{-4} M OP or a Zn to OP ratio of 1 to 4.

shown in Fig. 3 the presence of increasing concentrations of OP prevents the inactivation of the enzyme by 2.7×10^{-5} M $ZnCl_2$. The enzyme is fully protected at a ratio of [Zn^{++}]:[OP] of 1 to 4 (1.1×10^{-4} M OP).

Time Dependent Inhibition

Influence of Metal-binding Agents on Enzymatic Activity—Metal-binding agents also inhibit enzymatic activity when *preincubated* with the enzyme under suitable conditions of pH and temperature as shown in Table II. The preincubation of the enzyme with sodium sulfide, 2,3-dimercaptopropanol, sodium azide, OP, thiourea, diethyldithiocarbamate, disulfiram (Antabuse), 8-hydroxyquinoline, cupferron, and thyroxine results in a reduction of enzymatic activity. The degree of inhibition varies

TABLE II

Effect of preincubating metal-binding agents with glutamic dehydrogenase

The conditions of preincubation including concentrations of agent, pH, and time are indicated for the relative activity, V_i/V_c, shown. The temperature of incubation was 23°. In most instances the reaction was stopped at a time when approximately 50 per cent inhibition was achieved. Controls were treated in a similar fashion with omission of the metal-binding agent. The reaction tested was DPN → DPNH; the back reaction was studied for 1,10-phenanthroline and diethyldithiocarbamate and was inhibited to the same degree.

Agent	Concentration	pH incubation	Time incubation	V_i/V_c
	M		hrs.	
H_2S	Saturated	7.5	0.5	.08
Na_2S	5×10^{-3}	7.5	1.0	.50
2,3-Dimercaptopropanol	1×10^{-2}	7.0	1.0	.81
NaN_3	5×10^{-1}	7.0	72.	.50
1,10-Phenanthroline	2×10^{-3}	6.5	1.0	.52
Thiourea	5×10^{-1}	7.0	16.	.32
Diethyldithiocarbamate	1×10^{-1}	7.0	0.75	.51
Disulfiram	Saturated	7.0	60.	.50
8-Hydroxyquinoline	5×10^{-3}	7.0	90.	.50
Cupferron	2×10^{-1}	7.5	1.0	.50
Thyroxine	3×10^{-4}	7.5	3.0	.20

directly with the concentration of reagent, the temperature, and time of preincubation.

Nonreversibility of Time-dependent OP Inhibition—The time-dependent inhibition is *not* reversible. Preincubation of the enzyme with 1.5×10^{-2} M OP in phosphate buffer, pH 7.5, at 35° for 90 minutes, results in a reduction of a relative activity, V_i/V_c, to 0.78. A 2-fold dilution of OP in this mixture, while all other components are held constant, results in a relative activity, V_i/V_c, of 0.75. Dialysis of this mixture against 500 volumes of 0.1 M phosphate buffer overnight at 23° or for 1 hour at 35° does not change V_i/V_c.

The addition of $ZnCl_2$ at one-third of the OP concentration to the dialysis sack before dialysis in order to form a complex with OP also does not affect the degree of inhibition. Therefore, although the *instantaneous* inhibition of the enzyme by OP is completely reversible, its inactivation is *not* reversible by dilution, dialysis, or the addition of zinc ions when OP is *preincubated* with the enzyme.

DISCUSSION

Beef liver glutamic dehydrogenase is inhibited by metal-binding agents, both when these are present in the enzyme mixture and when preincubated with the enzyme before assay. A similar inhibition of other metallodehydrogenases by metal-binding agents has been demonstrated (2, 3, 8). The inhibition of glutamic dehydrogenase by a number of agents forming complexes with metals has recently been confirmed in another laboratory (10).

The considerations pertinent to the use of certain inhibitors of enzymatic activity to estimate the functional significance of metal(s) isolated with a purified enzyme system have been described (4). All of the metal-binding agents employed in this study, although varying in molecular structure and configura-

tion, bind zinc ions, but, as is true of other chelating agents, they are not specific in their capacity to form complexes with one specific metal.

Free metal ions may also act as inhibitors of glutamic dehydrogenase. Olson and Anfinsen (9) have shown that it is inhibited by Fe^{++}, Zn^{++}, Hg^{++}, and Ag^{+} ions. It is presumed that under such conditions metal ions combine with reactive, negative groups in the enzyme surface which are also essential to activity (11). The inhibition of glutamic dehydrogenase by the very metal ion that is essential to the catalytic activity of the enzyme is not unique, since this phenomenon has been observed for a number of metalloenzymes (4).

Added metal ions successfully compete with the enzyme zinc for OP, thereby preventing the inhibition of the enzyme (Table I). Those metals which form the tightest complexes with OP afford the greatest protection; magnesium which has little affinity for OP does not prevent inhibition at all. Inhibition does not occur when the concentration of free OP is too low, *i.e.* when practically all the chelating agent is combined with the added free metal ions. This latter effect is demonstrated by increasing concentrations of $ZnCl_2$ in the presence of constant concentrations of OP (Fig. 2). The activity achieved is maximal when the ratio of $[Zn^{++}]$:[OP] is 1:3, the maximal number of OP molecules that may be bound tetrahedrally by Zn^{++}, which has a coordination number of 6 (12, 13). The neutralization of inhibitions of both Zn^{++} and OP through the formation of the $Zn(OP)_3$ complex supports the hypothesis that the inhibition of enzymes by metal-binding agents is due to their capacity to form complexes with metal atoms which are enzymatically active.

When the enzyme is exposed to metal-binding agents, two types of inhibition are observed similar to those noticed with yeast alcohol dehydrogenase (2, 3). The instantaneous inhibition by OP and EDTA is reversible on dilution similar to the combination of free zinc ions with OP and EDTA. This suggests that the initial step of inhibition is the formation of a completely dissociable enzyme-inhibitor complex, with the inhibitor bound to the enzyme, zinc, which has been demonstrated for liver and yeast alcohol dehydrogenase (2, 14).

In contrast to the interaction of free metal ions with chelating agents, much less is known about that of metal ions integrated into the protein structure. Direct spectrophotometric evidence for a *mixed complex*, protein:metal:OP, has been obtained for horse liver alcohol dehydrogenase which contains 2 gm. atoms of zinc per mole with OP binding to the zinc atom *in situ* in the enzyme (14). By contrast, similar experiments with carboxypeptidase demonstrate that OP removes zinc from this enzyme (15). Attempts to demonstrate spectra on the addition of OP to glutamic dehydrogenase were unsuccessful, since the light scattered by the large glutamic dehydrogenase molecule in the ultraviolet region precluded the precise measurements required. The chemical mechanism of the inhibition of the enzyme by OP therefore has not been susceptible to the same spectrophotometric approach.

The capacity of metal-chelating agents to inhibit glutamic dehydrogenase activity is apparently due to the ability of their chemical groups to form a complex with the *intrinsic* zinc of the enzyme. Extrinsic metal ions may, however, be contaminants both of enzyme, of reagents, and water employed to study inhibition. These extraneous metal ions may compete with the zinc atoms of the enzyme for the coordinative site of the ligand thereby decreasing its effective concentration and preventing the agent from inhibiting enzymatic activity (Table I). Since many metals inhibit enzymatic activity, activation may in fact result (16) by their formation of complexes and removal from the enzyme. The inhibition of the enzyme measured in the presence of both metals and chelating agent is consequently less than would be obtained by the addition of the metal-binding agents alone. These circumstances all suggest that it is the metal-combining sites of these agents which are responsible for their inhibitory effects.

The time-dependent inhibition of the enzyme by OP is irreversible by dilution, dialysis, or the addition of zinc ions. The irreversibility suggests a permanent alteration in the enzymatic site subsequent to the attachment of the metal-binding agent to the enzyme zinc atoms. A similar effect has also been observed with catecholase (17). This copper enzyme is inhibited by cyanide and diethyldithiocarbamate; the inhibition can be prevented by the addition of Cu^{++}, Fe^{++}, Co^{++}, and Mn^{++} ions simultaneous with or just after exposure of the oxidase to these reagents. However, the inactivation produced by continued exposure of the enzyme to diethyldithiocarbamate and cyanide could not be reversed by the addition of these metal ions.

Calculations to determine the stoichiometry of the molecular events of inhibition have been proposed (18) which are based on the tacit assumption, among others, that the enzyme-inhibitor complex is fully and freely reversible. The theory predicts log (inhibitor) concentration against activity curves. These predictions would not be applicable to the glutamic dehydrogenase-Zn-OP system since the time-dependent reaction is not reversible, accounting for the steep slope of the inhibition curves (Fig. 1). Caution might be advised in the application of such calculations to other systems for reasons similar to those prevailing here.

The demonstration that inhibiting concentrations of OP (5×10^{-3} M) can dissociate the large glutamic dehydrogenase unit of molecular weight 1,000,000 into subunits of 500,000 and 250,000 demonstrates that this agent can profoundly affect the structural integrity of this enzyme. The reversibility of this reaction has not been reported. DPNH also dissociates the large unit (19). Similar observations with OP on yeast alcohol dehydrogenase have also been made (14). Zinc is apparently essential to both the catalytic property and the structural integrity of the enzyme. Further studies are required to determine whether these functions are independent or interdependent variables.

The inhibition of glutamic dehydrogenase by OP qualitatively resembles that of yeast alcohol dehydrogenase (2, 3). Both the instantaneous, reversible, and the time-dependent, irreversible inhibitions with OP here reported have been noticed with yeast alcohol dehydrogenase (2, 3). This is in contrast to the alcohol dehydrogenase of horse liver with which only the instantaneous, reversible inhibition has been observed. Preincubation of the enzyme leads to no further loss of enzymatic activity. A study of the kinetics of the yeast alcohol dehydrogenase OP interaction has yielded a consistent model for the mechanism of action of this enzyme with respect to the zinc atoms and to the mode of its inhibition; some of the features of this mechanism seem to apply to glutamic dehydrogenase.

SUMMARY

Beef liver glutamic dehydrogenase is inhibited by metal-binding agents including sodium sulfide, sodium azide, 1,10-phenanthroline, diethyldithiocarbamate, 8-hydroxyquinoline, cupferron,

and thyroxine. Both an instantaneous and time-dependent inactivation with 1,10-phenanthroline are observed. The instantaneous inhibition by 1,10-phenanthroline is reversible; the instantaneous inhibition by diethyldithiocarbamate is not. The time-dependent inhibition by both these reagents is irreversible.

The instantaneous inhibition by 1,10-phenanthroline can be prevented by the addition of Zn^{++}, Cu^{++}, Cd^{++}, Fe^{++}, Co^{++}, Ni^{++}, and Mn^{++} to the reaction mixture. Mg^{++}, which does not form a tight complex with 1,10-phenanthroline, does not prevent inhibition. Conversely, the inhibition of glutamic dehydrogenase by zinc ions can be prevented by the addition of 1,10-phenanthroline. The interaction between the metal of the enzyme and metal-binding agents is consistent with observations on simple chelate systems.

REFERENCES

1. Adelstein, S. J., and Vallee, B. L., *J. Biol. Chem.*, **233**, 589 (1958).
2. Hoch, F. L., Williams, R. J. P., and Vallee, B. L., *J. Biol. Chem.*, **232**, 453 (1958).
3. Williams, R. J. P., Hoch, F. L., and Vallee, B. L., *J. Biol. Chem.*, **232**, 465 (1958).
4. Vallee, B. L., *Advances in Protein Chem.*, **10**, 317 (1955).
5. Vallee, B. L., and Hoch, F. L., *Proc. Natl. Acad. Sci. U. S.*, **41**, 327 (1955).
6. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M., *J. Biol. Chem.*, **191**, 447 (1951).
7. Olson, J. A., and Anfinsen, C. B., *J. Biol. Chem.*, **197**, 67 (1952).
8. Vallee, B. L., Hoch, F. L., Adelstein, S. J., and Wacker, W. E. C., *J. Am. Chem. Soc.*, **78**, 5879 (1956).
9. Olson, J. A., and Anfinsen, C. B., *J. Biol. Chem.*, **202**, 841, (1953).
10. Ito, K., *Osaka Deigaku Igaku Zasshi*, **9**, 1059 (1957); *Chem. Abst.*, **52**, 4726 (1958).
11. Klotz, I., in H. Neurath and K. C. Bailey (Editors), *The Proteins, Vol. I*, Academic Press, Inc., New York, 1953.
12. Kolthoff, I. M., Leussing, D. L., and Lee, T. S., *J. Am. Chem. Soc.*, **73**, 390 (1951).
13. McClure, J. H., and Blanks, C. V., U. S. Atomic Energy Commission Bulletin ISC-164, 1951.
14. Vallee, B. L., Symposium VIII, Proteins, Proceedings IVth. International Congress of Biochemistry, Vienna, Pergamon Press, London, 1958, in press.
15. Vallee, B. L., Rupley, J. A., Coombs, T. L. and Neurath, H., *J. Am. Chem. Soc.*, **80**, 4750 (1958).
16. Redetzki, H. E., and Nowinski, W. W., Nature, **179**, 1010 (1957).
17. Tenenbaum, L. E., and Jensen, H., *J. Biol. Chem.*, **147**, 27 (1943).
18. Smith, E. L., Lumry, R., and Polglase, W. J., *J. Phys. and Colloid. Chem.*, **55**, 125 (1951).
19. Frieden, C., *Biochim. et Biophys. Acta*, **27**, 431 (1958).

Transamination Reactions of Mammalian Cells in Tissue Culture*

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A wide variety of mammalian cells in culture have been shown to require thirteen amino acids for survival and growth (1–4). The nonessential amino acids, not supplied in the medium, are synthesized by the cells from other components of the medium. Glucose[1] and glutamine (5) have been shown to serve as sources of carbon. Since transamination is an important reaction in the interconversion and biosynthesis of amino acids, it became of interest to examine the range of transaminase systems in cultured cells. Both a human cervical carcinoma cell (strain HeLa) and a mouse fibroblast (strain L) are here shown to have active transaminating systems leading to the formation of alanine, glutamic and aspartic acids, glycine, phenylalanine, and tyrosine from their corresponding α-keto analogues.

EXPERIMENTAL

Growth and Preparation of Cell Suspensions and Cell-free Extracts—Cultures of the HeLa cell and mouse fibroblast were grown as cellular layers adherent to the glass surface of 1-liter Blake bottles overlaid with the basal medium containing thirteen amino acids, eight vitamins, salts, and glucose (6). The medium was supplemented with 5 per cent dialyzed human serum in the case of the HeLa culture, and with 5 per cent dialyzed horse serum in the case of the L strain.

After 5 to 7 days of incubation at 37°, the medium was decanted, and the adherent cell layer washed twice with cold Krebs-Ringers salt solution (7) and harvested by scraping into the salt solution. Cell-free extracts were prepared by sonic disruption of a 20 per cent cell suspension in 10 to 15 ml. of distilled water in a Raytheon 9 kc. sonic oscillator for 10 minutes, followed by centrifugation at 20,000 × g for 30 minutes. Such preparations contained 15 to 20 mg. of protein per ml. and maintained their transaminase activity for several weeks when stored at −15°. The protein contents of the extracts were determined both by a modification of the procedure of Lowry (8) and by the micro-Kjeldahl method. The experiments reported here were carried out with these extracts, preliminary experiments having shown them to be more active than whole cells.

Assay of Transaminase Activity—The reaction mixtures contained usually 10 μmoles of keto acid, 20 μmoles of amino acid, 25 μg. of crystalline pyridoxal phosphate, phosphate buffer (0.1 M) of pH 7.5 to 8.0, and 1 ml. of enzyme preparation in a total volume of 3 ml. After incubation for 3 hours at 37° the reaction was stopped by heating in a boiling water bath for 5 minutes; the sample was then cooled, and 0.2 ml. of 3 M acetate buffer (pH 4.9) was added. The precipitated protein was removed by centrifugation, and an aliquot of the supernatant fluid was removed for amino acid assay. Results are expressed in terms of μmoles of amino acid formed per ml. of enzyme preparation.

Assays of Amino Acids—Quantitative assay of amino acids formed was performed chromatographically as previously described (9). In addition, L-glutamic acid, L-glutamine, and L-aspartic acid were determined by measuring the CO_2 produced from the acids by decarboxylation with lyophilized *Clostridium perfringens* (10).

Glycine was estimated colorimetrically by the method of Alexander *et al.* (11), as modified by Christensen *et al.* (12).

Most of the L-amino acids used were obtained commercially and were tested chromatographically for purity. Crystalline pyridoxal phosphate was kindly provided by Drs. E. A. Peterson and H. A. Sober, National Cancer Institute, Bethesda, Maryland.

α-Keto acids were obtained commercially. Chromatographic examination of their 2,4-dinitrophenylhydrazones showed that they were free from other keto acids. Sodium glyoxylate was synthesized by a modification of the method of Metzler *et al.* (13). We are indebted to Dr. Leon Levintow, National Institutes of Health, for samples of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid. Isonicotinic acid hydrazide (isoniazid) was a gift of Dr. Benjamin Prescott, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

RESULTS

The ability of cell-free extracts of the HeLa cell to mediate the transfer of the α-amino group of 17 amino acids to pyruvate, oxaloacetate, α-ketoglutarate, glyoxylate, phenylpyruvate, and *p*-hydroxyphenylpyruvate is shown in Table I. Similar experiments with extracts of the mouse fibroblast are summarized in Table II. Glutamic acid was the most active amino donor in both cell lines. In contrast, threonine and cysteine were consistently either minimally active or completely inactive as amino donors. It is of interest that whereas the amino groups of lysine, histidine, and methionine were activated by the extracts derived from the L cells, these amino acids were relatively inactive in transamination mediated by extracts derived from the HeLa cells. NH_3 was completely inactive with both cell lines.

* A portion of this work was presented at the Forty-eighth Annual Meeting of the Federation of American Societies for Experimental Biology at Chicago.

[1] H. Eagle, K. A. Piez, V. I. Oyama, and R. Fleischman, unpublished observations.

TABLE I

Transaminase activity in extracts of HeLa cell

Amino donor	Amino acid formed from corresponding keto acid in 3 hours (μmoles*)					
	Alanine	Aspartic	Glutamic	Glycine	Phenyl-alanine	Tyrosine
L-Alanine		0	2.1	1.0	0.5	0
L-Arginine	0.3	0	0	0	0.2	0
L-Aspartic	0.6		5.3	0	0	0.1
L-Cysteine	0	0.6	0.8	0	0	0
L-Glutamic	2.8	15		0.5	0.8	1.2
L-Glutamine	1.3	1.7		0.4	0.6	0.2
L-Glycine	0.3	0	1.5		0	0
L-Histidine	0	0.6	0	0	0	0
L-Isoleucine	0.4	1.0	3.5	0	0	0
L-Leucine	0.4	0.7	3.6	0	0	0
L-Lysine	0	0	0	0	0	0
L-Methionine	0.6	0	0	0.5	0	0
L-Phenylalanine	0.2	0	0.3	0		0.4
L-Threonine	0	0	0	0.1	0	0
L-Tryptophan	0.3	0.9	0	0.4	0	0.8
L-Tyrosine	0.3		1.5	0.6	0	
L-Valine	0.4	0	3.5	0	0.2	0
NH_3	0	0	0	0	0	0

* Endogenous and nonenzymatic values (*e.g.* glyoxylate) have been subtracted from values given.

TABLE II

Transaminase activity in extracts of mouse fibroblast

Amino donor	Amino acid formed from corresponding keto acid in 3 hours (μmoles*)					
	Alanine	Aspartic	Glutamic	Glycine	Phenyl-alanine	Tyrosine
L-Alanine		1.2	4.6	1.2	0	0.2
L-Arginine	0.4	0.9	1.4	0.7	0	0.1
L-Aspartic	1.0		4.9	2.0	0.2	0.6
L-Cysteine	0	0	0	0	0	0.5
L-Glutamic	5.5	17.2		2.9	0.2	2.5
L-Glutamine	3.7	1.1		2.4	0.1	0.4
L-Glycine	0.4	0	0.7		0.1	0
L-Histidine	0.4	0.7	0.6	0.3	0	0.1
L-Isoleucine	0.3	1.8	1.5	0.6	0.1	0.4
L-Leucine	0.6	2.0	5.5	0.9	0	0.3
L-Lysine	0.4	2.0	0	0.6	0	0
L-Methionine	0.3	0	2.6	1.0	0.4	0.2
L-Phenylalanine	2.0	0.5	1.6	0		0.6
L-Threonine	0.2	0	0	0.2	0	0
L-Tryptophan	1.5	0	0.7	0.2	0.4	1.0
L-Tyrosine	0		1.2	0.3	1.6	
L-Valine	2.6	1.2	4.7	0.3	1.2	0.1
NH_3	0	0	0	0	0	0

* Endogenous and nonenzymatic values (*e.g.* glyoxylate) have been subtracted from values given.

One of the most active transaminase systems studied was that catalyzing the formation of aspartic acid from oxaloacetate and glutamic acid. Aspartic acid is nutritionally nonessential for all the culture cells studied in this laboratory, and is presumably synthesized by the cell from oxaloacetate produced via the tricarboxylic acid cycle (14).

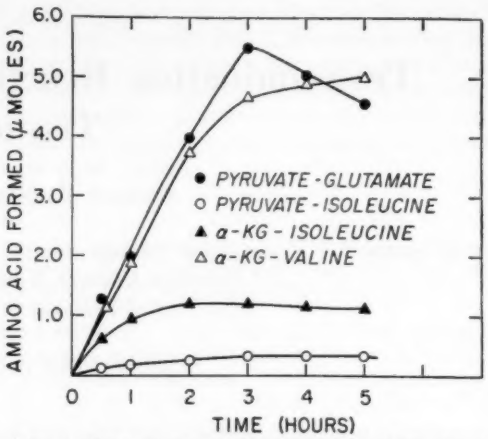

FIG. 1. Rate of transaminases of mouse fibroblast.

Glutamine is essential for the growth of these cultures (3), and is used by the cells for the synthesis of both protein and nucleic acid (5, 15). In a purified rat liver system, Meister *et al.* (16) have shown that glutamine rather than glutamic acid was active in transamination. However, Tables I and II show that glutamic acid was 9 to 16 times more effective than glutamine as an amino donor in transamination mediated by extracts from the L and HeLa cells, respectively. These results suggest that in these systems glutamine is first deamidated (5), and the resulting glutamic acid transaminated with the appropriate α-keto acid; the rate-limiting step in transamination presumably is the deamidation of glutamine.

Great variability was observed in the rates of transamination between different amino acids and α-keto acids (*cf.* Fig. 1). Thus, with pyruvate as the amino acceptor there were marked differences in rates between glutamate and isoleucine, and between isoleucine and valine when α-ketoglutarate was employed as the acceptor. Similar results were obtained with HeLa extracts. With both cell lines transamination reactions leading to the formation of arginine, valine, and leucine were either minimal or absent. The D isomers of the amino acids which were

TABLE III

Transamination with α-ketoglutarate and dipeptides and component amino acids with mouse fibroblast extract

The reaction mixtures contained 20 μmoles of dipeptide or component amino acid when used singly under the same conditions for transaminase assay as described in text.

Amino donor	L-Glutamate formed
	μmoles
L-Leucylglycine	6.6
Glycyl-DL-leucine	4.5
L-Leucylglycylglycine	6.8
L-Leucyl-L-tyrosine	3.7
D-Leucyl-L-tyrosine	1.0
Glycine	0.8
L-Leucine	7.3
D-Leucine	0
L-Tyrosine	1.2
Glycine* + L-leucine*	6.5

* 10 μmoles each.

tested, *i.e.* D-leucine, D-glutamate, D-phenylalanine, D-alanine, and D-aspartic acid were completely inactive as amino donors.

Transamination with Dipeptides—It has been shown that certain dipeptides can substitute for their corresponding essential amino acids for the growth of the mouse fibroblast and the HeLa cell (17). Table III shows that dipeptides were not so effective as amino donors as the component amino acids in the formation of glutamate catalyzed by extracts of the L cell. The reaction mixtures after incubation contained the component amino acids of the dipeptides, indicating that the extracts contained dipeptidases. It seems probable that hydrolysis of the dipeptides is a necessary precondition to their activity as amino donors.

Effects of Pyridoxal Phosphate and Isoniazid—Although pyridoxal phosphate has been shown to be a coenzyme of transaminase (18, 19), no effect was noted upon its addition in the various reactions studied with dialyzed cell-free extracts of the tissue cells. Presumably, the pyridoxal phosphate is firmly bound to the apoenzyme in the enzyme preparation employed. Isoniazid, which has been found to inhibit a number of enzyme systems including transaminases in bacteria (20, 21), had little or no effect on the transaminases of the tissue cell extracts studied.

DISCUSSION

The present studies have demonstrated the formation of glutamic and aspartic acids, phenylalanine, glycine, tyrosine- and alanine from the corresponding α-keto acids by transamina, tion reactions mediated by cell-free extracts of a human carcinoma cell and a mouse fibroblast. In growth experiments, when HeLa and L cells were incubated with isotopically labeled glucose, the nutritionally nonessential amino acids, glycine, serine, and alanine were heavily labeled;[1] and with C^{14}-labeled glutamine, glutamic and aspartic acids, proline, and asparagine were also labeled (5). The amino group of these nutritionally nonessential amino acids are presumably derived from other amino acids via transamination, since NH_3 is not utilized directly in amino acid biosynthesis (5). It is of interest that Wood and Cooley (22) were able to replace five amino acids essential for the growth of the rat with the corresponding α-keto acids; and Eagle and Oyama have shown that at least six keto analogues of essential amino acids will similarly support the growth of mammalian cell cultures.[1] Although certain dipeptides were active in transamination, this may be related to the fact that they were hydrolyzed under the conditions of the present experiments.

The failure of either pyridoxal phosphate and isoniazid to affect the transamination reactions may reflect the fact that the coenzyme is so firmly bound to the apoenzyme of the cell that it is not readily removed by dialysis or affected by the drug. It has been suggested (21) the inhibition of a number of enzyme systems by isoniazid may be due to competition between structurally related pyridoxal and isoniazid. Meister *et al.* (23) have shown that deoxypyridoxine phosphate, a known inhibitor of vitamin B_6, did not inhibit the apotransaminase when pyridoxal phosphate was firmly bound to the enzyme preparation. It is significant that Darnell and Levintow (24) have found that HeLa cells grown in vitamin B_6-deficient medium have diminished amounts of the nonessential amino acids in their intracellular pool.

SUMMARY

1. Cell free extracts of both the HeLa cell and mouse fibroblast form aspartic and glutamic acids, alanine, glycine, phenylalanine, and tyrosine by transamination of the corresponding α-keto acids with a variety of amino acids.

2. Glutamic and aspartic acids, leucine, isoleucine, and alanine were most active as amino donors. Other amino acids tested were weak or inert.

3. Glutamic acid was 9 to 16 times more effective than glutamine as an amino donor, and was the most active amino donor tested. These results suggest that glutamine may not, in fact, be involved in transamination until it is deamidated.

4. Several dipeptides were found to function in transamination reactions equally as effectively as the component amino acids. Since the extracts contained active dipeptidases, it is not clear whether the peptides *per se* or the constituent amino acids were the active compounds.

6. Pyridoxal phosphate and isoniazid had no appreciable effect on the transaminases.

REFERENCES

1. Eagle, H., *J. Biol. Chem.*, **214**, 839 (1955).
2. Eagle, H., *J. Exptl. Med.*, **102**, 37 (1955).
3. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., *J. Biol. Chem.*, **218**, 607 (1956).
4. Eagle, H., Oyama, V. I., and Levy, M., *Arch. Biochem. Biophys.*, **67**, 432 (1957).
5. Levintow, L., Eagle, H., and Piez, K. A., *J. Biol. Chem.*, **227**, 929 (1957).
6. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A. E., *J. Biol. Chem.*, **226**, 191 (1957).
7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and tissue metabolism*, Burgess Publishing Company, Minneapolis, 1949, p. 119.
8. Oyama, V. I., and Eagle, H., *Proc. Soc. Exptl. Biol. Med.*, **91**, 305 (1956).
9. Barban, S., *J. Bacteriol.*, **71**, 274 (1956).
10. Meister, A., Sober, H. A., and Tice, S. V., *J. Biol. Chem.*, **189**, 591 (1951).
11. Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, **160**, 51 (1945).
12. Christensen, H. N., Riggs, T. R., and Zweig, G., *Anal. Chem.*, **23**, 1521 (1951).
13. Metzler, D. E., Olivard, T., and Snell, E. E., *J. Am. Chem. Soc.*, **76**, 647 (1954).
14. Barban, S., and Schulze, H. O., *J. Biol. Chem.*, **222**, 665 (1956).
15. Salzman, N. P., Eagle, H., and Sebring, E. D., *J. Biol. Chem.*, **230**, 1001 (1958).
16. Meister, A., Sober, H. A., Tice, S. V., and Fraser, P. E., *J. Biol. Chem.*, **197**, 319 (1952).
17. Eagle, H., *Proc. Soc. Exptl. Biol. Med.*, **89**, 96 (1955).
18. Snell, E. E., *J. Am. Chem. Soc.*, **74**, 979 (1945).
19. Meister, A., Sober, H. A., and Peterson, E. A., *J. Am. Chem. Soc.*, **74**, 2385 (1952).
20. Gutman, N., Felton, S., and Huennekens, F. M., *Biochim. et Biophys. Acta*, **14**, 282 (1954).
21. Davison, A. N., *Biochim. et Biophys. Acta*, **19**, 131 (1956).
22. Wood, J. L., and Cooley, S. L., *Proc. Soc. Exptl. Biol. Med.*, **85**, 409 (1954).
23. Meister, A., Sober, H. A., and Peterson, E. A., *J. Biol. Chem.*, **206**, 89 (1954).
24. Darnell, J. E., and Levintow, L., *Federation Proc.*, **17**, 208 (1958).

ϵ-Aminocaproic Acid: an Inhibitor of Plasminogen Activation*

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Human and animal sera contain a globulin, plasminogen, which in the presence of activators is rapidly converted to plasmin, a proteolytic enzyme active at neutral hydrogen ion concentrations. Activators specific for plasminogen include streptokinase and staphylokinase of bacterial origin, urokinase and plasma activator found in body fluids, and fibrinokinase derived from tissues. Trypsin, an activator of other enzyme systems, will activate plasminogen, and plasminogen may undergo spontaneous activation. Streptokinase, urokinase, and trypsin activate plasminogen by a first order enzymatic reaction that involves the release of trichloroacetic acid soluble moieties (1), and spontaneous activation involves a similar process (2).

Plasmin acts on a number of substrates among which are casein, fibrin, fibrinogen, gelatin, protamine-heparin complex, and certain synthetic substrates containing lysine and arginine esters. Arginine and lysine esters are competitive inhibitors of the proteolytic activity of plasmin (3) as well as of the activator activity of urokinase, streptokinase, and trypsin (1). Numerous inorganic and organic inhibitors of plasmin activity have been described (4, 5) and often the same substances, which include toxic phosphorus compounds (6) also inhibit trypsin. However certain quaternary amines and laurylamine (5, 7), in high concentration, inhibit only plasmin and in low concentration possess the peculiar property of enhancing both the actions of plasmin and trypsin.

Hitherto, because of limitations of technical method, infrequent distinction has been made between plasmin inhibitors and inhibitors of plasminogen activation. The present communication describes the effects of ϵ-aminocaproic acid, a newly described "plasmin inhibitor" (8), as an inhibitor of plasminogen activation and also of plasmin. The results indicate that the primary action of ϵ-aminocaproic acid is to inhibit the activation of plasminogen, but that it also possesses, depending upon its concentration, the dual property of either inhibiting or enhancing the action of plasmin.

EXPERIMENTAL

Materials and Methods

ϵ-Aminocaproic Acid[1]—6-Amino hexanoic acid had a melting point of 208° (uncorrected) and on analysis was chemically pure.

Bovine Plasminogen—A bovine plasma Fraction III preparation,[2] containing 2.35 casein units of plasminogen per mg. of tyrosine (see below) was used as a source of bovine plasminogen. Activation with streptokinase was performed in the presence of trace amounts of a human plasminogen preparation (0.02 casein units per casein unit of bovine plasminogen).

Human Plasminogen—This was prepared by Kline's modification (9) of Christensen and Smith's (10) procedure from human plasma Fraction III.[3] The plasminogen preparations contained 100 to 150 casein units per mg. of tyrosine. These preparations have been characterized (11).

Human Plasmin—Human plasmin was prepared by spontaneous activation of human plasminogen (100 casein units per mg. of tyrosine) in 50 per cent glycerol (2). The activity of this preparation was 80 casein units per mg. of tyrosine.

Trypsin[4]—This was a salt free preparation crystallized two times.

Urokinase[5]—This contained 5100 Ploug units per mg. dry weight (12).

Fibrinokinase—Prepared by the method of Astrup and Sterndorff (13) this preparation, by the fibrin plate assay, showed an activity comparable to a urokinase solution containing 5.25 Ploug units per ml. or 84 Ploug units per mg. of tyrosine.

Streptokinase[1]—A highly purified preparation which was biophysically homogeneous was used. It contained 600 streptokinase units per mg. of nitrogen.

Phosphate Buffer—At 0.1 M, pH 7.6, this buffer was used in the assay procedures unless otherwise indicated.

Proteolytic Activity—Such activity was determined by a modification of the Remmert and Cohen (14) casein assay. Modification of the substrate concentration gave superior reproducibility. The casein[6] concentration in the digestion mixture was 2 per cent instead of the customary 4 per cent. A unit of activity released 180 μg. of tyrosine per hour; thus our unit is approximately 50 per cent of that originally described. Plasminogen was converted to plasmin by streptokinase, 1000 units per ml., a sufficiently large amount to insure almost instantaneous activation, and the plasmin was assayed by the same method.

Fibrin Plate Test—The fibrin plate assay (15) was modified so

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[1] Kindly supplied through Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, New York.

[2] Armour Research Division, Chicago, Illinois.

[3] Human plasma Fraction III was obtained from E. R. Squibb and Sons through the courtesy of the American Red Cross.

[4] Worthington Biochemical Corporation, Freehold, New Jersey.

[5] Obtained through the courtesy of Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen, Denmark.

[6] Hammarsten quality casein, Nutritional Biochemicals, Cleveland, Ohio.

that a 0.2 per cent solution of bovine fibrinogen[7] was used.[8] The fibrinogen was contaminated with bovine plasminogen, thereby rendering the fibrin plate susceptible to the action of plasminogen activators; this system has been widely used for the assay of plasminogen activators (16). The product of two perpendicular diameters of the lysed zone was used as a measure of proteolytic or activator activity, but the lysed area is not directly proportional to the amount of enzyme employed. A linear relationship is seen when the logarithm of the enzyme concentration is plotted against the logarithm of the activity (17).

Streptokinase cannot be directly assayed on bovine plasminogen, since bovine plasminogen preparations do not contain proactivator (16, 18). Before the assay of the streptokinase solutions they were mixed with 0.01 casein unit of human plasminogen per ml. to supply proactivator (this trace amount of added plasminogen was insufficient to affect the plate assay, which at its lower limit is sensitive to 0.1 casein unit of plasmin per ml.).

Kinetic Studies in Presence of ε-Aminocaproic Acid—Studies on the activation of plasminogen in the presence of ε-aminocaproic acid involved the determination of the amount of plasmin activated in a given period of time. ε-Aminocaproic acid and the activator being studied interfered with the plasmin assay and had to be removed before assay. The activation reaction was stopped by lowering the pH in the activation mixture to 2.0 at which pH plasmin is stable. The ε-aminocaproic acid was removed by dialysis in the cold against 0.01 N hydrochloric acid. The activator was removed or denatured by precipitation of the plasmin with 1 M sodium chloride at pH 2.0 (18), and the washed precipitate was dissolved in distilled water and then assayed.

Determination of Esterase Activity—This was done by the hydrolysis of benzoyl arginine methyl ester (3). Of the material to be assayed, 0.2 ml. was added to 0.2 ml. of buffer plus 6 ml. of benzoyl arginine methyl ester (0.5 mg. per ml. in phosphate buffer, 0.05 M, pH 7.6). After 2 minutes and 32 minutes of incubation at 37°, 3-ml. specimens of the digestion mixture were withdrawn and immediately added to 0.1 ml. of 10 per cent acetic acid, thus stopping the digestion process by lowering the pH. The optical density of the 32-minute specimen was read in the Beckman spectrophotometer at wave length 258 mμ, (19) with the 2-minute specimen used as blank value. Plasminogen was assayed after conversion to plasmin.

RESULTS

Inhibition of Plasminogen Activation by ε-Aminocaproic Acid—Fig. 1 shows that ε-aminocaproic acid inhibited the enzymatic process (1) involved in plasminogen activation by streptokinase rather than the proteolytic activity of plasmin itself. The experimental conditions were bovine plasminogen, 3.2 casein units per ml.; streptokinase, 1000 units per ml.; and trace amounts of human plasminogen to provide proactivator. In one series, varying concentrations of ε-aminocaproic acid were added at the start of the activation period (30 minutes at 37°), and in the other series ε-aminocaproic acid was added at the end of the activation period and immediately before plasmin assay. The results in Fig. 1 and in later figures have been expressed as percentage inhibition of control activity.

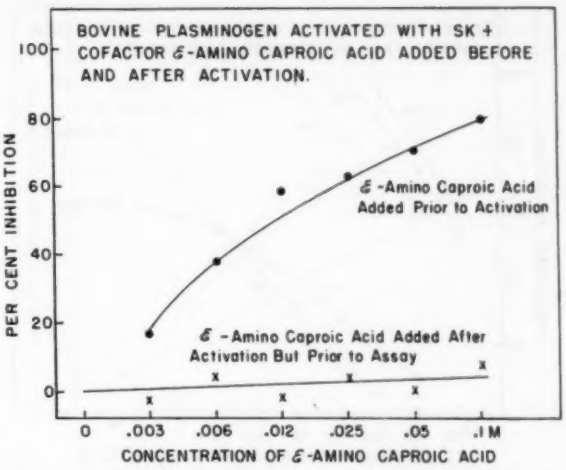

FIG. 1. The ordinate indicates per cent inhibition of the control assay value and the abscissa, the concentration of ε-aminocaproic acid plotted on a logarithmic scale. The effect of ε-aminocaproic acid was to inhibit the activation process without significant inhibition of plasmin. SK, streptokinase.

The addition of ε-aminocaproic acid, at the end of the activation period, did not affect the assay values for plasmin[9] (bottom line of Fig. 1), but its addition at the start of the activation period caused an inhibition of the activation process, the degree of which was a function of the added ε-aminocaproic acid concentration (top line of Fig. 1). Precisely similar findings were recorded in another experiment, in which human plasminogen was substituted for bovine plasminogen.

Fibrin Plate Assay—The effect of ε-aminocaproic acid upon trypsin and plasmin and upon the plasminogen activators, streptokinase, fibrinokinase, and urokinase, was studied by means of the fibrin plate test. The respective enzyme concentrations were adjusted to give, in the absence of ε-aminocaproic acid, roughly similar zones of plate lysis. Fig. 2 demonstrates the degree of inhibition of each enzyme (in per cent) plotted against the concentrations of added ε-aminocaproic acid.

Concentrations of ε-aminocaproic acid which inhibited the three plasminogen activators had little or no effect on plasmin and trypsin activities. There was a 60-fold difference between the concentrations of ε-aminocaproic acid causing 50 per cent inhibition in the two test systems, the figures being 0.5 M for trypsin and approximately 0.008 M for the activators.

Kinetics of Inhibition of Proteolytic Activity—The kinetics of the inhibition of the proteolytic activity of plasmin by ε-aminocaproic acid were studied, with casein as a substrate. Fig. 3 shows the inhibition of plasmin activity (0.49 casein unit per ml. in digestion mixture) at two concentrations of ε-aminocaproic acid. The casein concentration was varied 7-fold. The data are plotted with the reciprocal of the substrate concentration

[7] Armour Laboratories, Kankakee, Illinois.

[8] Some batches of fibrinogen clotted poorly and partially disintegrated during the incubation period. This difficulty was overcome by dissolving the fibrinogen in borate buffer (0.1 M, pH 8.0) containing calcium chloride (0.14 per cent) and dextran (1.2 per cent). Plates prepared in this manner were stable and exhibited greater sensitivity than those prepared by the original technique.

[9] The ineffectiveness of ε-aminocaproic acid as a plasmin inhibitor, in the concentrations shown in Fig. 1, was due in part to the 5-fold dilution of the activation mixture made for assay purposes.

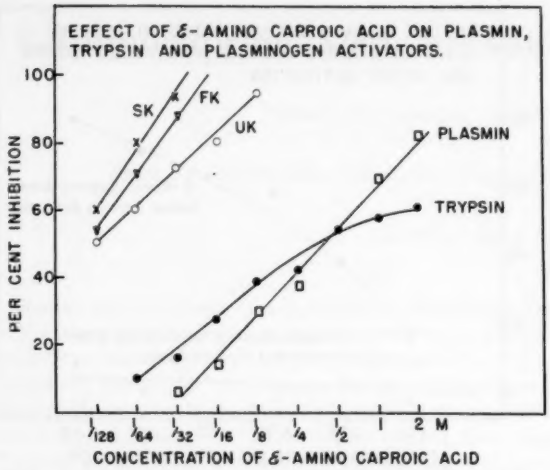

FIG. 2. Fibrin plate assays. The concentrations of materials tested were adjusted to give equal zones of lysis in the absence of ϵ-aminocaproic acid. Marked differences are apparent between the inhibitory effect exerted by ϵ-aminocaproic acid on streptokinase (SK), fibrinokinase (FK), and urokinase (UK) on the one hand and plasmin and trypsin on the other.

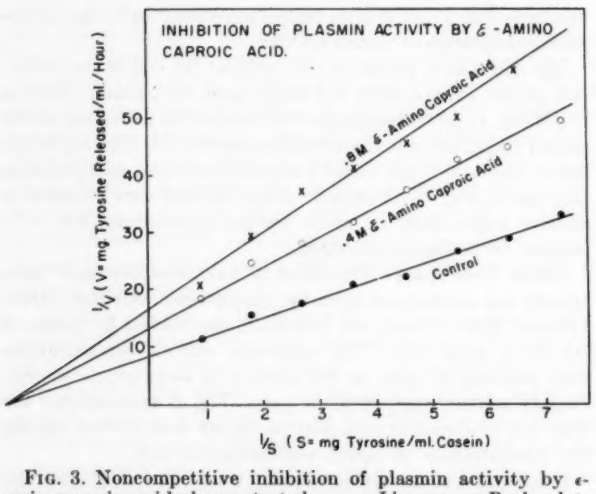

FIG. 3. Noncompetitive inhibition of plasmin activity by ϵ-aminocaproic acid demonstrated on a Lineweaver-Burk plot. The highest casein concentration in the digestion mixture was 2 per cent, containing 1.1 mg. of tyrosine per ml. Substrate concentration (S) is expressed as mg. of casein tyrosine per ml. of digestion mixture, since the assay procedure used determined tyrosine rather than casein concentration.

as abscissa, and the reciprocal of the reaction velocity as ordinate as suggested by Lineweaver and Burk (20). The double reciprocal plot revealed that ϵ-aminocaproic acid inhibited the proteolytic activity of plasmin in a noncompetitive fashion.

Similar observations were made with trypsin used in place of plasmin. The final concentration of trypsin in the digestion mixture was 2 μg. per ml.; the noncompetitive inhibition of the proteolytic activity of trypsin by ϵ-aminocaproic acid is illustrated in the double reciprocal plot in Fig. 4.

Kinetics of Inhibition of Activator Activity—ϵ-Aminocaproic acid inhibited the activation of plasminogen by urokinase (Fig.

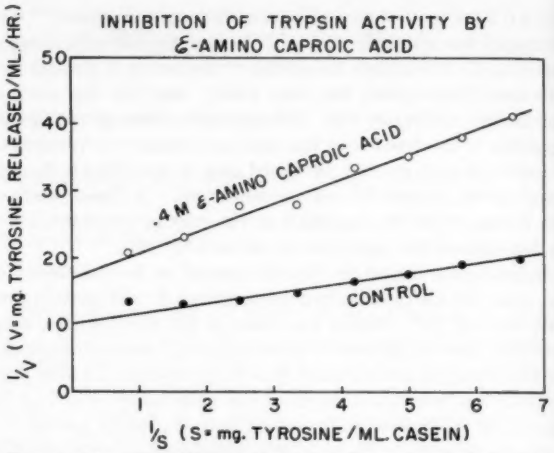

FIG. 4. Noncompetitive inhibition of trypsin activity by ϵ-aminocaproic acid demonstrated on a Lineweaver-Burk plot. S is expressed in terms of tyrosine for the reasons noted in Fig. 3.

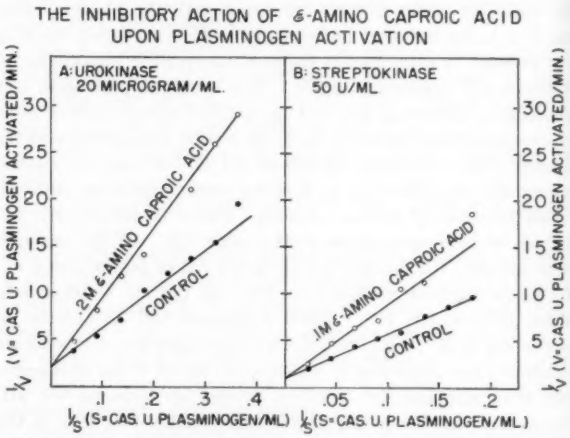

FIG. 5. Lineweaver-Burk plots demonstrating that the inhibitory action of ϵ-aminocaproic acid upon urokinase and streptokinase was competitive in nature. CAS. U., casein units.

5*A*). The highest plasminogen concentration was 22 casein units per ml., and the concentration was varied 7-fold. The plasmin formed was assayed by casein hydrolysis after removal of ϵ-aminocaproic acid and urokinase, and complete recovery of plasminogen and plasmin was demonstrated by tyrosine assays. The results plotted in the double reciprocal manner fulfill the Lineweaver-Burk criteria for competitive inhibition.

Fig. 5*B* illustrates a precisely similar experiment except that streptokinase was used as an activator. The plasminogen concentration in this experiment varied from 6 to 48 casein units per ml. and the streptokinase concentration was 50 units per ml. The results, presented in a double reciprocal plot, show that the ϵ-aminocaproic acid inhibited plasminogen activation by streptokinase in a competitive manner.

Because of technical reasons the plasminogen concentrations in the latter two experiments were high, and since ϵ-aminocaproic acid was a competitive inhibitor, a correspondingly high inhibitor concentration had to be used in order to demonstrate the inhibitory action. However, complete inhibition resulted

with lower concentrations of ε-aminocaproic acid when lower concentrations of plasminogen and activator were used (Fig. 2).

The demonstration that tryptic activity as measured by proteolytic assay was inhibited noncompetitively by ε-aminocaproic acid (Fig. 4) strongly suggested that ε-aminocaproic acid would inhibit the activation of plasminogen by trypsin in a similar fashion. The data illustrated in Fig. 6 provide confirmation of this hypothesis. It was impracticable to test the activator properties of plasmin in this manner, since the activation reaction proceeds so slowly that reliable kinetic data cannot be obtained. Nevertheless, since plasmin activity, measured by proteolytic assay, was inhibited noncompetitively by ε-aminocaproic acid (Fig. 3), there can be little doubt that a similar relationship would hold for plasmin activator activity.

Enhancing Effect of ε-Aminocaproic Acid on Proteolytic Activity—The experiments thus far described have revealed that ε-aminocaproic acid, in concentrations exceeding 0.03 M, acted as a noncompetitive inhibitor of the proteolytic activities of trypsin and plasmin. However, there were indications that ε-aminocaproic acid in certain lower concentrations would increase the plasmin activity above that found in the control solution containing no ε-aminocaproic acid. Plasmin assays were made in the presence of varying concentrations of ε-aminocaproic acid with three different substrates: casein, fibrin, and benzoyl arginine methyl ester. The plasmin concentration was 2.4 casein units per ml., and the ε-aminocaproic acid concentration was varied from 5×10^{-4} M to 2 M; in the actual digestion mixture the ε-aminocaproic acid concentration was varied from 1.6×10^{-5} M to 1 M, the dilution depending upon the assay system. The results are presented in Fig. 7, where the increase (or decrease) in activity (measured in per cent of the control value) is plotted against the ε-aminocaproic acid concentration in the digestion mixture.

With all three substrates enhanced proteolytic activity was demonstrated with a peak value at an ε-aminocaproic acid concentration of 10^{-2} M. The peak percentage of enhancement was approximately the same for each substrate, and the range over which an increase was found was roughly the same for benzoyl arginine methyl ester and casein. The range of ε-aminocaproic acid concentrations which caused increased proteolysis on the fibrin plate was somewhat narrower than for casein and benzoyl arginine methyl ester, but here the true concentration of the enzyme-ε-aminocaproic acid mixture varied, since the lysed area increased during the incubation period, thus diluting the solution which was being assayed.

Effect on Solubility of Plasminogen and Plasmin—An incidental observation was made in the course of this investigation, namely, that addition of ε-aminocaproic acid increased the solubility of plasminogen and plasmin.[10] Ordinarily plasminogen prepared by acid extraction, and plasmin prepared from this plasminogen, were but sparingly soluble at neutral pH, but when ε-aminocaproic acid was present in the solvent, even in low concentrations, plasminogen and plasmin formed a clear solution. It was also noted that when precipitation of plasminogen or plasmin by 1 M NaCl at pH 2.0 was attempted, an ε-aminocaproic acid concentration of as little as 0.01 M was sufficient to prevent complete precipitation. ε-Aminocaproic acid also increased the solubility effect on casein in the presence of 5 per cent trichloroacetic acid.

[10] F. B. Ablondi and J. J. Hagan (personal communication) have made similar observations with regard to lysine and certain other basic amino acids.

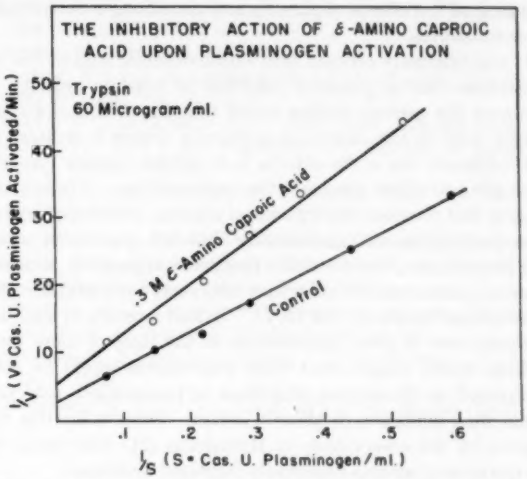

FIG. 6. A Lineweaver-Burk plot illustrating that the inhibitory effect of ε-aminocaproic acid upon the activation of plasminogen by trypsin is noncompetitive in nature. Cas. U., casein units.

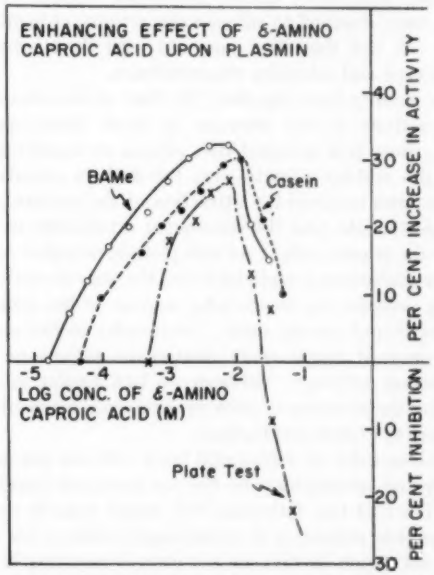

FIG. 7. ε-Aminocaproic acid in low concentration enhances the action of plasmin upon benzoyl arginine methyl ester (BAMe), casein and fibrin (plate test).

This effect was not seen when the concentration of ε-aminocaproic acid was 0.2 M or less; however, at higher concentrations, prior dilution of the solution allowed for complete precipitation.

DISCUSSION

Numerous substances have been described as inhibitors of plasmin (3–7), but in most instances no distinction has been made between inhibition of the enzyme itself and inhibition of the activation process. Such a distinction is now possible because of advances in purification of the enzyme and its precursor, increasing knowledge of the activation process, and a better under-

standing of the effects of plasmin and plasminogen activators on their substrates.

It was originally claimed that ϵ-aminocaproic acid in low concentrations was a powerful inhibitor of plasmin activity (8). However the present studies reveal that the action of ϵ-aminocaproic acid on the plasminogen-plasmin system is complex and that although one of its actions is to inhibit plasmin, its effect upon the activation process is the more striking. These studies suggest that previous descriptions of plasmin inhibitors may have been incomplete, and particularly that the quaternary amines and laurylamine, which exhibit properties apparently similar to those of ϵ-aminocaproic acid, may also exert their primary effect upon the activation process (5, 7). Indeed a review of analytical methods used in these publications in the light of more recent findings would suggest that these experiments would be better interpreted as illustrating inhibition of plasminogen activation rather than inhibition of plasmin action. Support for this view is given by the observation by Bjerrehuus (21) who found that the quaternary amines apparently inhibited urokinase.

The mechanism for the enhanced activity of plasmin observed with certain concentrations of ϵ-aminocaproic acid is obscure. It is unlikely that the solvent action of ϵ-aminocaproic acid upon plasmin or some of its substrates will account for this phenomenon, since quaternary amines in similar concentration (10^{-2} M) have been observed to enhance the activity of highly soluble plasmin (7), and the effect was seen with three substrates of different type and solubility characteristics.

It has recently been reported (22) that cholinesterase has increased activity in the presence of short chain quaternary amines. Since it is accepted that plasma cholinesterase has an anionic site and an esteratic site, the authors speculated that the mechanism involved the attraction of the quaternary amine to the anionic site, and the consequent attachment of the substrate to the esteratic site. As with plasmin at higher concentrations, the quaternary amines inhibited the enzyme activity (23), a finding perhaps due to blocking because of the proximity of the esteratic and anionic sites. Noteworthy in this connection is the failure of longer chain quaternary amines to enhance cholinesterase activity. Although we lack similar information concerning the structure of plasmin, analogous mechanisms may be invoked to explain our findings.

Differences exist on a temporal basis between the actions of urokinase and streptokinase on the one hand and trypsin on the other. The first two activators will, under suitable conditions, cause complete activation of plasminogen within a few minutes, whereas activation by trypsin is a process requiring hours. In this respect activation by trypsin is comparable to the autocatalytic activation of plasminogen itself, although this latter mode of activation is even slower than with trypsin. These temporal differences have suggested that activation may take place in at least two ways, a suggestion supported by the fact that study of trypsin activation or autocatalytic activation requires the use of a stabilizing agent of which glycerol has been the most useful (2). In previous communications (1, 2) we have reported that the activation of plasminogen occurs by consequence of the release of trichloroacetic acid-soluble moieties, and that it appeared that the amounts of such moieties released varied with the mode of activation. Biophysical evidence (11) tended to support this finding. Thus it is not surprising that an apparently specific inhibitor of the plasminogen activation process should exert a predominant effect upon one class of activators rather than upon the other. Urokinase and streptokinase are both inhibited competitively and at low ϵ-aminocaproic acid concentration (under the conditions customarily used); however, trypsin and plasmin (the "activator" present during the autocatalytic process) are inhibited noncompetitively and at high ϵ-aminocaproic acid concentrations. These findings are held to confirm our former suggestion that at least two kinds of active plasmin molecules exist, showing probably identical biochemical activity, but differing molecular sizes. Analogous findings have been reported with regard to the activation of prothrombin, which results in molecules of similar biological activity and varying molecular weight (24).

It has been previously reported (18, 25) that lysine and ornithine serve, respectively, to inhibit the activation of plasminogen by streptokinase and by the plasma activator obtained from human corpses after sudden death from anoxemia. The observation that ϵ-aminocaproic acid (lysine without the α-amino group) suppresses competitively plasminogen activation by specific activators (streptokinase, urokinase, and tissue activator) raises considerations concerning the structural requirements involved in the activation site, also with respect to the development of additional competitive inhibitors.

The clear differences existing between the modes of inhibition produced by ϵ-aminocaproic acid upon activators and upon plasmin can be turned to practical use. Where, as in plasma and serum, both activities may occur together, the use of ϵ-aminocaproic acid to inhibit plasminogen activator at concentrations insufficient to affect plasmin activity, permits differentiation of their separate effects.[11, 12]

SUMMARY

1. ϵ-Aminocaproic acid competitively inhibited the activation of human or bovine plasminogen by streptokinase, urokinase, and probably fibrinokinase, but inhibited plasminogen activation by trypsin noncompetitively.

2. ϵ-Aminocaproic acid in concentrations exceeding 0.06 M was a noncompetitive inhibitor of the proteolytic activities shown by plasmin or trypsin. In lower concentrations it enhanced the proteolytic action of plasmin.

3. The results support the view that plasminogen activation may occur by two mechanisms yielding plasmins with similar biochemical activities but of different molecular size.

Addendum—In a recent communication to the American Chemical Society ("Streptokinase activation of plasminogen: species specificity and inhibition by ϵ-aminocaproic acid," Chicago, September 1958), F. B. Ablondi and J. J. Hagan have independently studied the inhibitory effect of ϵ-aminocaproic acid on plasminogen activation. Their results (personal communication) are qualitatively in agreement with our own.

[11] N. Alkjaersig, A. P. Fletcher, and S. Sherry, submitted for publication.

[12] S. Sherry, R. I. Lindemeyer, A. P. Fletcher, and N. Alkjaersig, submitted for publication.

REFERENCES

1. Alkjaersig, N., Fletcher, A. P., and Sherry, S., *J. Biol. Chem.*, **233**, 86 (1958).

2. Alkjaersig, N., Fletcher, A. P., and Sherry, S., *J. Biol. Chem.*, **233**, 81 (1958).

3. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.*, **208**, 85 (1954).
4. Geiger, W. B., *J. Immunol.*, **69**, 597 (1952).
5. Astrup, T., and Alkjaersig, N., *Nature*, **169**, 314 (1952).
6. Mounter, L. A., and Shipley, B. A., *J. Biol. Chem.*, **231**, 855 (1958).
7. Astrup, T., and Alkjaersig, N., *Nature*, **168**, 565 (1951).
8. British Patent Specification, 770,693. Her Majesty's Stationery Office, London, 1957.
9. Kline, D. L., *J. Biol. Chem.*, **204**, 949 (1953).
10. Christensen, L. R., and Smith, D. H., *Proc. Soc. Exptl. Biol. Med.*, **74**, 840 (1950).
11. Shulman, S., Alkjaersig, N., and Sherry, S., *J. Biol. Chem.*, **233**, 91 (1958).
12. Ploug, J., and Kjeldgaard, N. O., *Biochim. et Biophys. Acta*, **24**, 278 (1957).
13. Astrup, T., and Sterndorff, I., *Acta Physiol. Scand.*, **36**, 250 (1956).
14. Remmert, L. F., and Cohen, P. P., *J. Biol. Chem.*, **181**, 431 (1949).
15. Astrup, T., and Mullertz, S., *Arch. Biochem. Biophys.*, **40**, 346 (1952).
16. Astrup, T., *Blood*, **11**, 781 (1956).
17. Astrup, T., and Alkjaersig, N., *Arch. Biochem. Biophys.*, **37**, 99 (1952).
18. Troll, W., and Sherry, S., *J. Biol. Chem.*, **213**, 881 (1955).
19. Schwerdt, G. W., and Takenaka, Y., *Biochim. et Biophys. Acta*, **16**, 570 (1955).
20. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
21. Bjerrehuus, I., *Scand. J. Clin. and Lab. Invest.*, **4**, 179 (1952).
22. Erdos, E. G., Foldes, F. F., Zsigmond, E. K., Baart, N., and Zwartz, J. A., *Science*, **128**, 92 (1958).
23. Bergman, F. In *The physical chemistry of enzymes*, The Faraday Society, Aberdeen, Scotland, 1955, p. 126.
24. Seegers, W. S., and Alkjaersig, N., *Arch. Biochem. Biophys.* **61**, 1 (1956).
25. Mullertz, S., *Proc. Soc. Exptl. Biol. Med.*, **85**, 326 (1954).

Properdin: Preparation from Plasma Fraction I of the Cohn Method*

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Pillemer *et al.* (1) have shown that normal human and other mammalian serums contain a trace protein which has been called properdin. Properdin, in conjunction with complement and Mg^{++}, participates in the natural defense mechanism of blood (2).

Pillemer *et al.* (1) have described a method for preparing properdin from serum. This method employs zymosan to adsorb the properdin, the trace protein being eluted with high ionic strength ($\mu = 0.6$) saline. This method has been modified by Pennell *et al.* (3), starting with plasma obtained from either acid-citrate-dextrose or resin-collected blood.

Rothstein and Pennell (4) have reported the purification of properdin from Fraction I eliminating the use of zymosan. Pondman and Prins (5) have obtained purified properdin from serum utilizing ion-exchange techniques.

In an effort to prepare large quantities of purified properdin for clinical evaluation, a number of fractions obtained from the Cohn cold ethanol process (6) were investigated. During our work with these fractions, we were informed by the Protein Foundation Laboratories that they had found properdin activity in Cohn's Fraction I. This observation was confirmed here and Fraction I has indeed proven to be a rich source of properdin (7).

In these laboratories a glycine-citrate buffer extraction step is employed to purify the fibrinogen in Fraction I. Considerable properdin activity was found in this extract and when purified by the methods described in this paper, the glycine-citrate buffer extract yielded high potency properdin.

EXPERIMENTAL

Methods and Materials

The zymosan assay method of Pillemer *et al.* (8) was used to determine the properdin content of the fractions reported here. Aliquots for assay were frozen immediately and maintained at −20°, then shipped in dry ice via air freight to Mr. Earl Todd at Western Reserve University. All values reported here have been determined by Mr. Todd.

A normal human serum was frozen in 2-ml. aliquots and stored at −20°. A vial of this control serum was frequently submitted for properdin assay under a coded label. No significant variations in properdin levels were reported over an 8-month period for this serum.

All nitrogen values reported here have been obtained by the semimicro-Kjeldahl method and have been corrected for nonprotein nitrogen content.

Fraction I was obtained from citrated normal human plasma by the method of Cohn *et al.* (6). The yields of Fraction I averaged about 4.2 kg. from 300 l. of citrated plasma.

The glycine-citrate buffer utilized to extract the properdin from Fraction I was similar to the buffer employed by Blombäck and Blombäck (9) for the purification of fibrinogen. The composition of the buffer used here consisted of 0.0055 M sodium citrate, 0.233 M sodium chloride, 0.058 M sodium acetate, 1.0 M glycine, and 7 per cent ethanol. The final solution was adjusted to pH 6.0 with 1 N acetic acid.

The yields of all precipitates obtained here are reported as wet weights and are used as such.

Extraction—Fraction I was suspended in 10 volumes (weight per volume) of cold (−3°) glycine-citrate buffer with the aid of a Waring Blendor. The resulting suspension was then stirred for 2 hours at −3° and the fibrinogen removed by low temperature (−3°) centrifugation.

The supernatant liquid was stored at −5° for 3 to 5 days in order to precipitate a fibrinogen-like material which contained little, if any, properdin activity. This precipitate was discarded after removal by centrifugation at −5°.

Zinc Precipitation—A properdin-rich fraction was precipitated from the supernatant liquid just described by raising the temperature of the solution to 2° and then adding sufficient 1.0 M zinc acetate to make the final solution 0.02 M in zinc (20 ml. per l.). The zinc acetate solution was added slowly with vigorous stirring and the final solution then stored at 2° for 18 hours.

After storage at 2°, the zinc-protein precipitate was collected by centrifugation at the same temperature and dissolved in 5 volumes (weight per volume) of 0.25 M ethylenediaminetetraacetic acid which had been adjusted to pH 7.4. Approximately 0.5 hour of mechanical stirring at room temperature was necessary to accomplish this. This solution was then dialyzed against 0.15 M sodium chloride at 2° to remove most of the zinc and ethylenediaminetetraacetic acid. Two changes of 50 volumes each of saline were used over a 72-hour period.

Removal of Inert Proteins with Tetrametaphosphate—The dialyzed solution was further purified by adding a 10 per cent solution of sodium tetrametaphosphate (Cyclophos)[1] (10) to give a final concentration of 1 per cent (weight per volume), followed by a pH adjustment to 4.2 with 5 per cent HCl. Considerable inert material precipitated and the solution was placed at 2° overnight to insure completeness of precipitation. After 18 hours at 2° the precipitate was removed by centrifugation. Occasionally the supernatant liquid gave assay results indicating a 50 per cent

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[1] Available under the trade name "Cyclophos" from the Victor Chemical Works, Chicago, Illinois.

loss, but on ethanol precipitation assay values repeatedly indicated essentially all of the activity had remained in the tetrametaphosphate supernatant liquid. More recently Nitschmann *et al.* (11) have used polymetaphosphates to fractionate human plasma proteins.

Ethanol Precipitation—Properdin of a still higher purity and in a more concentrated and usable form was obtained by ethanol precipitation. This was accomplished by first adjusting the pH of the tetrametaphosphate supernatant liquid to 6.8 by the addition of 1 M $NaHCO_3$. An equal volume of cold (2°) distilled water was then added and the resulting solution cooled to the freezing point. After the formation of a light slush, 50 per cent ethanol (precooled to −20°) was added slowly with vigorous stirring until the final ethanol concentration reached 20 per cent by volume. The temperature of the solution was not permitted to exceed 0° during the ethanol addition. The ethanolic solution was then permitted to stand overnight at −5°. On the following morning (18 hours), the precipitated properdin was collected by low temperature (−5°) centrifugation.

Since the presence of ethanol is undesirable for experiments *in vivo*, the 20 per cent ethanol precipitate was usually suspended in 100 to 200 ml. of cold distilled water and lyophilized. After drying, the lyophilized powder, or in some cases the ethanol paste, was dissolved in a volume of cold saline-phosphate buffer (0.15 M NaCl, 0.05 M PO_4, pH 7.4) equivalent to 10 to 20 times the weight (weight per volume) of the ethanol precipitate. This solution was then clarified by centrifugation at 2° and the supernatant liquid containing the properdin decanted.

Sterile properdin solutions have been prepared by filtration of this saline-phosphate solution through a sterile Republic S-6 pad.[2] Some loss in activity has been seen, although essentially no difficulties have been encountered in preparing sterile, nontoxic, pyrogen-free solutions of properdin by the methods just described.

Additional Purification—Properdin of a still higher purity was obtained by subjecting the final solution described above (before sterile filtration) to a second treatment with sodium tetrametaphosphate followed by ethanol precipitation in identical fashion as applied to the dialyzed ethylenediametetraacetic acid solution.

RESULTS AND DISCUSSION

The process just described has been easily reproduced and has given uniform results with typical values as shown in Table I. Only a few properdin preparations were continued through the second tetrametaphosphate step. Table II illustrates the yields and specific activities obtained when tetrametaphosphate is employed a second time.

Stability—Purified properdin solutions obtained from Fraction I have exhibited no gross instability when stored at low temperatures (2°) for several days. Frozen (−20°) solutions have maintained full potency for weeks. Heating at 56° for 30 minutes, however, destroys essentially all activity.

Toxicity—The acute intravenous LD_{50} in mice of one properdin preparation was greater than 3000 units per kg.[3] The same preparation has been given intravenously to human patients in quantities as high as 4000 units per kg. of body weight over a 2-hour period with no visible signs of toxicity.[4]

[2] Republic Seitz Filter Corporation, 17 Stone Street, Newark, New Jersey.

[3] Samuel McKinney and Harold M. Peck, unpublished observations (1957).

[4] Chester M. Southam, unpublished observations (1958).

TABLE I

Purification of properdin

Fraction	Volume and/or weight	Properdin	Properdin	Total properdin units
		units/ml.	*units/mg. protein N*	
Citrated human plasma	600 l.			
Zinc precipitate	358 gm.			
Dialyzed ethylenediaminetetraacetic acid solution	3.38 l.	100	57.2	3.38×10^5
Tetrametaphosphate supernatant liquid	3.46 l.	50	290	1.73×10^5
20 per cent alcohol precipitate	10 gm. (144 ml.)	2000	826	2.88×10^5

TABLE II

Second purification of properdin with tetrametaphosphate

Fraction	Volume	Properdin	Properdin	Total properdin units
	ml.	*units/ml.*	*units/mg. protein N*	
20 per cent alcohol precipitate	50.0	2000	826	1×10^5
Tetrametaphosphate supernatant liquid	53.0	1750	1540	0.93×10^5
Tetrametaphosphate precipitate	23.6	100	110	0.024×10^5

TABLE III

Ultracentrifugal analysis of properdin

Component	$s^0_{20,w}$	Per cent
1	1.54	26
2,3	6.0	70
4	18.1	4

Purity—The specific activities of properdin preparations derived from Fraction I have been equal to or greater than those of preparations obtained in these laboratories by the zymosan method of Pillemer *et al.* (1).

In the analytical ultracentrifuge[5] (Spinco model E), properdin isolated from Fraction I revealed the presence of four components when examined in saline-phosphate buffer at pH 7.4 (Table III). Pillemer (12) has indicated that native properdin is an extremely high molecular weight substance (24 to 30 S). The amount of such high molecular weight material in properdin from Fraction I is very small and preliminary preparative ultracentrifuge experiments have associated the greater portion of the activity with components 2 and 3 (5 to 6 S). Isliker[6] has essentially confirmed Pillemer's observations with native properdin in human serum and with zymosan-prepared material. However, Isliker has observed the dissociation of these large molecules when exposed to cysteamine to give over 90 per cent of a component with a sedimentation coefficient of 6 S.

It is quite possible that properdin from Fraction I has under-

[5] Analysis by Dr. Donald Williams.

[6] H. C. Isliker, personal communication (1958).

gone a dissociation during ethanol fractionation as suggested by Isliker.

SUMMARY

Properdin activity has been found in Fraction I of plasma separated by the Cohn cold ethanol method. A procedure for preparing highly purified properdin from Fraction I has been described. The probability that this properdin is a dissociation product of native properdin has been indicated. Properdin activity has been purified over 2000-fold, assuming that the original plasma contained 8 units of properdin per ml., or 0.7 unit per mg. of protein nitrogen.

REFERENCES

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. A., and Wardlaw, A. C., *Science*, **120**, 279 (1954).
2. Wardlaw, A. C., and Pillemer, L., *J. Exptl. Med.*, **103**, 553 (1956).
3. Pennell, R. B., Rothstein, F., Surgenor, D. M., Eyquem, A., and Todd, E. W., *Proc. Soc. Exptl. Biol. Med.*, **96**, 273 (1957).
4. Rothstein, F., and Pennell, R. B., *Abstracts VI^e Congrès de la Société Européene d'Hématologie*, Longjaers Bogtrykkeri, Copenhagen, 1957, p. 293.
5. Pondman, K. W., and Prins, H. K., *Abstracts VI^e Congrès de la Société Européene d'Hématologie*, Longjaers Bogtrykkeri, Copenhagen, 1957, p. 294.
6. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459 (1946).
7. Sanders, B. E., Spicer, D. S., and Smith, E. V. C., *Voz Sanguinis*, **3**, 16 (1958).
8. Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., and Todd, E. W., *J. Exptl. Med.*, **103**, 1 (1956).
9. Blombäck, B., and Blombäck, M., *Arkiv Kemi*, **10**, 415 (1956).
10. Rane, L., and Newhouser, L. R., U. S. Patent No. 2,726,235, 1955.
11. Nitschmann, H. S., Rickli, E., and Kistler, P., *Abstracts IV international congress of biochemistry*, Section 16, No. 21, Pergamon Press, London, 1958, p. 194.
12. Pillemer, L., *Ann. N. Y. Acad. Sci.*, **66**, 233 (1956).

The Synthesis and Some Properties of Amino Acyl Adenylates*

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Recent investigations have provided evidence for the enzymatic activation of amino acids according to the following scheme in which an enzyme-bound amino acyl adenylate is formed by reaction of amino acid, adenosine 5′-triphosphate, and a specific amino acid activating enzyme:

$$\text{Amino acid} + \text{ATP} + \text{enzyme} \rightleftharpoons \text{enzyme-amino acyl adenylate} + \text{pyrophosphate} \quad (1)$$

This reaction is analogous to that catalyzed by the acetate activating system (2) and is consistent with observations of amino acid-dependent enzymatic pyrophosphate-adenosine 5′-triphosphate exchange (3–5). Studies in several laboratories (6–11) have provided evidence supporting the enzymatic formation of amino acyl adenylates. Direct evidence for the enzymatic formation of such anhydrides has been obtained in this laboratory by Karasek *et al.* (12), who isolated a compound with the properties of tryptophanyl adenylate from an enzymatic reaction mixture; independent experiments of a similar nature (13–15) also support the concept that amino acyl adenylates are formed according to Reaction 1. It is possible that such anhydrides occur and react only in an enzyme-bound form, and are therefore not in equilibrium with an external source of anhydride. However, under appropriate conditions, several synthetic acyl adenylates have been found to be enzymatically active, *e.g.* acetyl adenylate (2), hexanoyl adenylate (16), propionyl adenylate (17), phenylacetyl adenylate (18), benzoyl adenylate (18, 19), amino acyl adenylates (10–12, 20). The possibility that amino acyl adenylates may be intermediates in the incorporation of amino acids into proteins (5), and perhaps therefore in protein synthesis, suggests the need for studies on the chemical and biochemical properties of these anhydrides.

The present report is concerned with the preparation and the study of certain properties of several amino acyl adenylates. Previously, DeMoss *et al.* (20) have prepared amino acyl adenylates by condensing amino acid chlorides with silver adenosine 5′-phosphate, and Berg (21) has obtained several amino acyl adenylates by condensing free amino acids with adenosine 5′-phosphate in the presence of *N*,*N*′-dicyclohexylcarbodiimide (22). Preparations of amino acyl adenylates are extremely unstable and rapidly undergo hydrolysis and other reactions including conversion to a compound which appears to be the amino acid ester of the 2′ (or 3′) hydroxyl group of adenosine 5′-phosphate. In this paper we describe the preparation of amino acyl adenylates via the corresponding *N*-carbobenzoxy amino acids. This procedure, which appears to have certain advantages over the other methods, was described briefly in a preliminary communication (1). A similar method has been employed independently by Zioudrou *et al.* (23) for the synthesis of tyrosinyl adenylate and glycyltyrosinyl adenylate. The condensation of *N*-carbobenzoxy amino acid with adenosine 5′-phosphate in the presence of *N*,*N*′-dicyclohexylcarbodiimide proceeds smoothly and in good yield, and the resulting carbobenzoxy amino acyl adenylate is relatively stable during purification procedures. The carbobenzoxy group is readily removed by catalytic hydrogenation (24). We have applied this procedure to the preparation of 15 amino acyl adenylates. Glycyl adenylate and tryptophanyl adenylate, prepared from radioactive amino acids, have been employed in studies with rat liver preparations capable of incorporating amino acids into proteins.

EXPERIMENTAL

Materials—The amino acids, AMP and *N*,*N*′-dicyclohexylcarbodiimide were obtained from Schwarz Laboratories, Inc. Inosinic acid was obtained from Sigma Chemical Company. Carbobenzoxy chloride and the carbobenzoxy amino acids were prepared as described by Bergmann and Zervas (24). Pyridine ("spectro grade") was purchased from Eastman Kodak Company. Ethylene glycol monomethyl ether was obtained from Howe and French, Inc., and fractionated through a short Vigreux column (b.p. 123–4°). The carbobenzoxy amino acyl hydroxamic acids and amino acyl hydroxamic acids were prepared by reacting the corresponding methyl or ethyl esters with methanolic hydroxylamine essentially as described (25). It was not possible to obtain satisfactory preparations of the α-hydroxamic acids of glutamine and asparagine (or their *N*-carbobenzoxy derivatives) by these procedures. Benzoyl adenylate was prepared as previously described (18). Palladium catalyst was prepared by dissolving 10 gm. of palladium black (Fisher Scientific Company) in aqua regia and evaporating to dryness at 128°. The residue was dissolved in concentrated hydrochloric acid and then evaporated again; this procedure was repeated twice. The residue was dissolved in the minimal volume of hot concentrated hydrochloric acid and poured rapidly into boiling water; 10 ml. of 88 per cent formic acid were added to the boiling suspension followed by sufficient 5 N potassium hydroxide to make the solution alkaline to litmus paper. After addition of 0.2 ml. of 88 per cent formic acid, the solution was cooled and the catalyst was washed several times with water by decantation.

Studies with Rat Liver Preparations—The rat liver preparations were obtained by differential centrifugation of a 25 per cent rat liver homogenate in a buffered salt-sucrose medium as described

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† Postdoctoral research fellow of the National Heart Institute, National Institutes of Health.

TABLE I

Properties of carbobenzoxy amino acyl adenylates

Amino acid	AMP*	N†		Ionophoretic mobility‡		$R_F \times 100$§	Hydroxamic acid $R_F \times 100$ Solvent‖		
		Calculated	Found	Borate	Tris		a	b	c
				mm.	*mm.*				
L-Alanine	0.955	15.2	15.2	+110	+55	72	98	94	96
β-Alanine	0.973	15.2	15.5	+111	+54	68	84	90	85
L-Asparagine	0.948	16.5	16.5	+117	+69	69	82	81	88¶
L-Glutamine	0.941	16.1	15.7	+115	+51	73	78	79	78¶
Glycine	1.01	15.6	15.1	+103	+57	76	87	86	86
L-Isoleucine	0.986	14.1	14.8	+98	+58	80	100	96	94
L-Leucine	1.02	14.1	14.0	+103	+50	82	99	97	94
L-Methionine	1.03	13.7	13.4	+98	+54	77	98	97	92
L-Phenylalanine[a]	1.08	13.4	13.6	+100	+45	81	97	96	94
L-Proline	1.00	14.5	14.8	+114	+63	79	95	83	89
L-Serine	0.934	14.8	15.1	+106	+52	79	79	76	81
L-Threonine	1.01	14.4	14.6	+109	+65	72	87	90	79
L-Tryptophan[a]	1.14	14.7	14.3	+88	+41	80	96	95	95
L-Tyrosine	1.02	13.0	12.7	+102	+48	75	98	94	94
L-Valine	0.968	14.5	14.2	+109	+50	77	90	96	87

* Moles of AMP per gm. formula weight of product, based on absorption at 260 mμ. The compounds were dissolved in 0.1 ml. of water and treated with 0.6 ml. of 2 M hydroxylamine hydrochloride (adjusted to pH 6.5 with NaOH); after standing for 10 minutes at 26°, the solutions were diluted with water and read at 260 mμ in a Beckman ultraviolet spectrophotometer. Carbobenzoxy tryptophan gave 25 per cent of the absorption of AMP at this wave length; the other carbobenzoxyamino acids did not absorb significantly under these conditions.

† Dumas.

‡ Determined with the apparatus of Markham and Smith (32); Whatman No. 3 paper; 0 to 5°; 20 volts per cm., 0.1 M; tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.1) or 0.1 M sodium borate buffer (pH 9.1). The values given in the table describe the position of the ultraviolet-quenching band after 80 minutes; the values for AMP were +169 mm. (borate) and +136 mm. (Tris). (See Fig. 1.)

§ Ascending paper chromatography on Whatman No. 1 paper at 5° for 18 hours; the solvent consisted of ethylene glycol monomethyl ether and water (75:25); the $R_F \times 100$ value for AMP was 54.

‖ Ascending paper chromatography on Whatman No. 1 paper (24–28°); the compounds (1 to 2 μmoles) were treated with 0.1 ml. of 4 M hydroxylamine, and 0.01 ml. of this solution was applied to the chromatogram. Solvent a: isobutanol, formic acid, water (75:15:10); solvent b: *n*-butanol, acetic acid, water (4:1:1); solvent c: ethanol, water (80:20).

¶ Standard compounds were not available for comparison.

[a] The corresponding D-amino acid derivatives exhibited similar properties.

by Zamecnik and Keller (26). In certain experiments the reaction mixtures were centrifuged after incubation to obtain the microsome and supernatant fractions (27). The proteins were precipitated by addition of perchloric acid and processed by a modification of the procedures of Hulbert and Potter (28) and Tyner *et al.* (29). The proteins were dissolved in 2 N sodium hydroxide at 50–60° and reprecipitated by addition of trichloroacetic acid. The reprecipitated proteins were washed twice with 5 per cent trichloroacetic acid and twice with 95 per cent ethanol before determination of radioactivity (30).

Methods—The hydroxamic acid determinations were carried out by the general procedure of Lipmann and Tuttle (31). The sample (0.2 to 0.5 ml.) was treated with 0.5 ml. of 2 M hydroxylamine hydrochloride (adjusted to pH 6.5 with potassium hydroxide), and after 5 minutes, 1.5 ml. of ferric chloride reagent (0.37 M ferric chloride, 0.2 M trichloroacetic acid, 0.67 N hydrochloric acid) were added; the final volume was brought to 2.5 ml. with water. The colors were read in a Weston photometer equipped with a 535 mμ filter.

Carbobenzoxy-L-alanyl Adenylate—AMP (685 mg.; 2 mmoles) and carbobenzoxy-L-alanine (446 mg.; 2 mmoles) were dissolved in 10 ml. of 75 per cent aqueous pyridine and the solution was cooled to 0°. A solution prepared by dissolving 8 gm. of *N,N'*-dicyclohexylcarbodiimide in 8 ml. of pyridine was added rapidly and the mixture was shaken at 0 to 5° for 2 hours. The precipitate of dicyclohexyl urea, which formed gradually during the reaction, was removed by filtration at 5°, and washed with 3 ml. of cold 75 per cent pyridine. On standing at 5° for 2 to 10 minutes, the combined filtrate and washing separated into two layers. The upper layer was discarded, and the lower layer, which contained more than 90 per cent of the hydroxamic acid-forming material present in the original reaction mixture, was added to 200 ml. of acetone at −15°. The precipitate was washed by centrifugation with 2 portions of 200 ml. of acetone. The precipitate was drained for a few minutes and then stirred for 5 minutes with 50 ml. of ethylene glycol monomethyl ether. The precipitate was removed by centrifugation and the extraction with ethylene glycol monomethyl ether was repeated.[1] The ex-

[1] The carbobenzoxy amino acyl adenylate is soluble in ethylene

tracts were combined and mixed with 500 ml. of ethyl ether. The precipitate of carbobenzoxy-L-alanyl adenylate was washed twice with ether and dried at 26° in a vacuum over P_2O_5 for 30 minutes, and stored at −15°.

$C_{21}H_{23}O_{10}N_6P$, (carbobenzoxy-L-alanyl adenylate)

Calculated: C 45.7, H 4.5, N 15.2, P 5.6
Found: C 45.4, H 4.7, N 15.2, P 5.4

Other properties of this compound are given in Table I. The other carbobenzoxy amino acyl adenylates were obtained in a similar manner; the yields varied from 60 to 90 per cent.

Properties of Carbobenzoxy Amino Acyl Adenylates—Paper chromatography of the carbobenzoxy amino acyl adenylates (Table I) revealed that these preparations did not contain appreciable quantities of free AMP; under the chromatographic conditions employed, a 5 per cent impurity of free AMP could have been detected. Treatment of the carbobenzoxy amino acyl adenylates with hydroxylamine gave hydroxamic acids which moved together with the authentic carbobenzoxy amino acyl hydroxamic acids in three paper chromatographic systems. The total nitrogen values were in good agreement with the calculated values (Table I). Determinations of AMP based on the absorption at 260 mμ were close to the theoretical value of 1.00 (1.25 for carbobenzoxy tryptophanyl adenylate; see footnote, Table I). The carbobenzoxy amino acyl adenylates were hydrolyzed in 0.1 N sodium hydroxide (10 minutes, 26°) and the hydrolysates were analyzed for AMP with adenylic acid deaminase (33); in each case, the values obtained from absorption at 260 mμ were confirmed. The analytical data and the fact that the carbobenzoxy amino acyl adenylates moved as homogeneous compounds on paper chromatograms and paper ionophoresis (see below), are consistent with a relatively high degree of purity. The products were 85 to 95 per cent pure on the basis of the hydroxylamine-ferric chloride procedure; as discussed below, quantitative reaction with hydroxylamine may not be assumed to occur under the conditions of this procedure.

Paper ionophoretic studies at pH 9.1 revealed that all of the carbobenzoxy amino acyl adenylates moved toward the positive electrode with a mobility which was somewhat less than that of AMP (Fig. 1). Both AMP and the carbobenzoxy amino acyl adenylates exhibited greater mobility in the presence of borate (Table I). These findings are consistent with the proposed structures, *i.e.* an anhydride linkage between the carboxyl group of the amino acid and the phosphoric acid group of AMP. The increase of ionophoretic mobility in borate appears to exclude esterification of the ribose hydroxyl groups of AMP. Furthermore, periodate titrations carried out as described by Schmidt *et al.* (34) revealed that the carbobenzoxy amino acyl adenylates consumed stoichiometric quantities of periodate; after reaction with periodate, treatment with hydroxylamine gave the corresponding carbobenzoxy amino acyl hydroxamic acids which were identified by paper chromatography.

Acylation of the amino group of adenine was excluded by reaction of the carbobenzoxy amino acyl adenylates with nitrous acid. Thus, 14 mg. of carbobenzoxy-L-tryptophanyl adenylate were dissolved in 3.5 ml. of 1.8 M acetic acid and treated with 80 mg. of sodium nitrite. The absorption maximum gradually shifted from 260 mμ to 249 mμ; the reaction proceeded to 28, 50, and 71 per cent of completion in 0.5, 1, and 2 hours, respectively at 26°. After standing for an additional 16 hours at 5°, the reaction reached 96 per cent of completion; the quantitative hydroxamic acid reaction did not change significantly, and carbobenzoxy tryptophanyl hydroxamic acid was identified by paper chromatography.

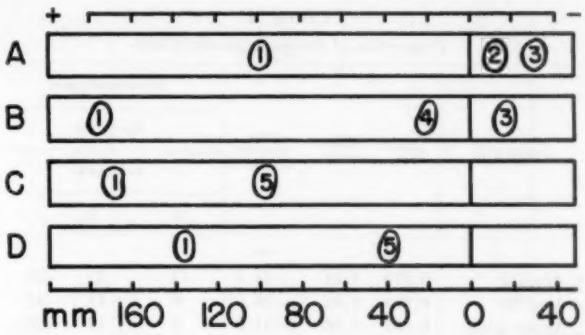

FIG. 1. Paper ionophoresis on Whatman No. 3 paper with the apparatus of Markham and Smith (32), 0 to 5°, 20 volts per cm. *A*. 0.05 M ammonium formate buffer; pH 4.5, 120 minutes; *B*. 0.01 M sodium citrate buffer; pH 5.9, 120 minutes; *C*. 0.1 M sodium borate buffer; pH 9.1, 80 minutes; *D*. 0.1 M tris(hydroxymethyl)-aminomethane buffer; pH 9.1, 80 minutes. Key: 1, AMP; 2, L-tryptophan; 3, L-tryptophanyl adenylate; 4, adenylic acid-2′(3′)-L-tryptophanate; 5, carbobenzoxy-L-tryptophanyl adenylate. In *A*, 3 and 4 moved together; in *B*, 2 and 3 moved together. In *A*, inosine and L-tryptophanyl inosinate moved with 2 (see the text).

Carbobenzoxy-L-tryptophanyl inosinate was also prepared by condensing inosinic acid with carbobenzoxy-L-tryptophan by the procedure described above for the preparation of carbobenzoxy amino acyl adenylates. *N*, calculated for $C_{29}H_{29}O_{11}N_6P$; 12.6; found, 12.0. The yield of carbobenzoxy-L-tryptophanyl inosinate was 20 per cent of theory; the low yield may be ascribed, at least in part, to the low solubility of inosinic acid in aqueous pyridine. The product was converted to L-tryptophanyl inosinate by catalytic hydrogenation as described below. The final product and its *N*-carbobenzoxy derivative gave the corresponding hydroxamic acids on treatment with hydroxylamine.

Preparation of Amino Acyl Adenylates—The carbobenzoxy amino acyl adenylate (150 to 250 mg.) was suspended in 5 ml. of 90 per cent acetic acid and stirred with a magnetic stirrer. After the material had dissolved, the solution was cooled to 0° and approximately 1 gm. (wet weight) of freshly prepared palladium catalyst was added. The reaction vessel was flushed with nitrogen for 3 minutes, and then hydrogen was admitted to the system. The reaction was complete in 10 to 30 minutes. After removal of the catalyst, this solution was lyophilized. The dry powder was washed with anhydrous ether and placed in a vacuum over phosphorus pentoxide at −15°. Some properties of amino acyl adenylates prepared by this procedure are given in Table II. The over-all yield of amino acyl adenylates varied from 40 to 81 per cent, based on the free amino acids. Thus, in the synthesis of glycyl-1-C^{14}-adenylate, a yield of 326 mg. (81 per cent) was obtained from 75 mg. of glycine-1-C^{14}. DL-Tryp-

glycol monomethyl ether whereas significant quantities of AMP are not extracted. Usually two extractions were sufficient; with certain derivatives, *e.g.* carbobenzoxy asparaginyl adenylate and carbobenzoxy glutaminyl adenylate, three or four extractions were necessary to obtain most of the product in solution. The extraction procedure was followed by hydroxamic acid tests on the residual solid material and on the extract.

TABLE II

Properties of amino acyl adenylates

Amino acid	AMP*	Amino acid†	Iono-phoretic mobility‡	Hydroxamic acid $R_F \times 100$ Solvents§		
				a	b	c
			mm.			
L-Alanine	0.970	1.01	−34.4	13	32	66
β-Alanine	0.974	1.02	−38.0	9	18	45
L-Asparagine	0.981	1.06	−31.8	7	12	26‖
L-Glutamine	1.02	0.972	−36.8	8	42¶	37‖,¶
Glycine	1.01	0.958	−34.7	7	13	43
L-Isoleucine	1.05	1.01	−29.4	51	63	78
L-Leucine	0.980	0.982	−29.2	67	63	78
L-Methionine	0.940	0.89	−34.9	65	63	62[b]
L-Phenylalanine[a]	0.976	0.972	−36.4	55	63	73
L-Proline	1.02	0.980	−38.7	18	23	55
L-Serine	0.988	1.07	−33.6	7	12	42
L-Threonine	1.02	0.959	−33.0	11	22	58
L-Tryptophan[a]	1.18	0.958	−30.2	42	46	54
L-Tyrosine	1.04	0.968	−31.6	(19,33)[c]	(29,55)[c]	65
L-Valine	0.988	1.01	−33.7	35	57	73

* Moles of AMP per gm. formula weight of product, based on absorption at 260 mμ. The compounds were dissolved in 0.7 ml. of 2 M hydroxylamine hydrochloride (adjusted to pH 6.5 with NaOH); after standing for 10 minutes at 26°, the solutions were diluted with water and read at 260 mμ in a Beckman ultraviolet spectrophotometer. Tryptophan gave 24 per cent of the absorption of AMP at this wave length; the other amino acids did not absorb significantly under these conditions.

† Moles of amino acid per gm. formula weight of product determined by the method of Moore and Stein (35); proline was determined as described by Troll and Lindsley (36).

‡ Conditions were as described in footnote ‡, Table I, except that 0.05 M ammonium formate buffer (pH 4.5) was used; value for AMP was +100 mm.; time, 2 hours. (See Fig. 1.)

§ See footnote ‖, Table I, for conditions.

‖ Standard compounds were not available for comparison.

¶ A spot corresponding to γ-glutamylhydroxamic acid was also observed.

[a] The corresponding D-amino acid derivatives exhibited similar properties.

[b] The values for the major spots are reported; in each solvent, a smaller spot corresponding to carbobenzoxy methionyl hydroxamic acid and a faint unidentified spot were also observed.

[c] This derivative and authentic tyrosinyl hydroxamic acid gave two spots with these solvent systems.

tophanyl-3-C^{14}-adenylate was obtained in 40 per cent over-all yield.

Properties of Amino Acyl Adenylates—Treatment of the amino acyl adenylate preparations with hydroxylamine followed by paper chromatography gave hydroxamic acids (located by spraying with ferric chloride), which exhibited R_F values identical to those of the corresponding standard amino acid hydroxamic acids (Table II). The marked difference in chromatographic behavior between the amino acid hydroxamic acids (Table II) and the carbobenzoxy amino acid hydroxamic acids (Table I) made it possible to detect the presence of carbobenzoxy amino acyl adenylates in the corresponding amino acyl adenylate preparation. All of the amino acyl adenylates appeared free from the corresponding carbobenzoxy derivatives except for some preparations of methionyl adenylate. Larger quantities of catalyst were needed to remove completely the carbobenzoxy group of carbobenzoxy methionyl adenylate; however, considerable desulfuration occurred with large amounts of catalyst.

The lyophilized amino acyl adenylate preparations gave AMP and amino acid values which were close to theory (Table II). Several amino acyl adenylate preparations (D-tryptophanyl adenylate, D-phenylalanyl adenylate, L-methionyl adenylate, L-tryptophanyl adenylate) were hydrolyzed in 0.1 N sodium hydroxide and examined for the presence of the corresponding enantiomorphic amino acids by optically-specific enzymatic methods (37); no racemization of the amino acids was found under conditions suitable for the detection of 0.5 μmole of D- or L-amino acid in the presence of 100 μmoles of the corresponding optical isomers.

Determinations of amino acyl adenylates by the hydroxylamine-ferric chloride procedure gave color values which were 60 to 95 per cent of those obtained with standard hydroxamic acids. We observed some variability in these values even with products obtained from the same carbobenzoxy amino acyl adenylate preparations. In general, lower values were obtained when longer periods of hydrogenation were employed. Some hydrolysis undoubtedly occurs during hydrogenation, lyophilization, and storage at −15°. However, it cannot be assumed that the amino acyl adenylates react quantitatively with hydroxylamine to yield hydroxamic acids under the conditions employed. It has been found that certain compounds with activated acyl groups react with the hydroxyl group of hydroxylamine to yield unstable derivatives that give no color with ferric chloride (38). The extent of such reactions is dependent upon several factors including the pH (39). As one might expect, the rate of reaction with hydroxylamine varies greatly with different carboxyl derivatives of a given amino acid and with a given derivative of different amino acids (39, 40).

On paper ionophoresis at pH 4.5, the amino acyl adenylates moved toward the negative electrode (Fig. 1 and Table II). Under the conditions employed, the amino acyl adenylates were separated from free amino acid and AMP. The observed mobility of the amino acyl adenylates is consistent with the proposed anhydride structures, which would be expected to have a net positive charge at pH 4.5. L-Tryptophanyl inosinate exhibited a mobility of 11.4 mm. in the direction of the cathode; under these conditions, an identical value was observed with inosine. Since neither of these compounds would be expected to have an appreciable net positive charge at pH 4.5, it seems probable that the observed mobility was due to electroendosmosis.

The α-amino acyl adenylates were rapidly hydrolyzed to AMP and amino acid in neutral aqueous solution as judged by disappearance of the ability to form hydroxamic acids; however, they exhibited greater stability at lower values of pH (Fig. 2). Under conditions similar to those employed in enzymatic studies, *i.e.* 0.1 M potassium phosphate buffer (pH 7.2) and 37°, the α-amino acyl adenylates were rapidly hydrolyzed, whereas the carbobenzoxy amino acyl adenylates and benzoyl adenylate, were relatively stable. β-Alanyl adenylate was hydrolyzed much more slowly than were the α-amino acyl adenylates (Fig. 3).

It is of interest that the curves for the disappearance at pH 7.2 of hydroxamic acid-forming material (Fig. 3) obtained with certain α-amino acyl adenylate preparations did not reach zero

even after 2 hours. On the other hand, complete loss of the hydroxamic acid reaction was observed in 2 to 5 minutes at pH 9.5. Incubation of amino acyl adenylates at 37° and pH 7.2 for 1 hour, followed by treatment with hydroxylamine and paper chromatography, revealed the presence of the corresponding amino acyl hydroxamic acids. In freshly prepared samples of amino acyl adenylates, the relatively stable hydroxamic acid-forming fraction usually represented about 5 to 10 per cent of the total color values. On storage at −15° for several weeks the relatively stable fraction increased in amount; the rate of conversion varied with different amino acyl adenylates. Such conversion was not associated with a marked decrease in the total hydroxamic acid values, or with changes in AMP to amino acid ratios and ionophoretic mobility at pH 4.5. However, paper ionophoresis at pH 5.9 in 0.01 M sodium citrate buffer gave evidence for the formation of a new compound. As indicated in Fig. 1, paper ionophoresis at pH 5.9 separated L-tryptophanyl adenylate from the new compound, which moved toward the positive electrode.[2] Preparations of L-prolyl adenylate, L-threonyl adenylate, and L-tryptophanyl adenylate that had been stored for several months were subjected to paper ionophoresis at pH 5.9, and the negatively charged materials were separated from free AMP and free amino acid and eluted from the paper. The eluted compounds gave the corresponding amino acyl hydroxamic acids on treatment with hydroxylamine, but did not react with periodate. Hydrolysis with 0.1 N sodium hydroxide (1 hour at 26°) gave equimolar quantities of AMP and amino acid. The evidence strongly suggests that the products formed from the corresponding amino acyl adenylates are the 2′(or 3′)-amino acid esters of AMP. All of the α-amino acyl adenylates were active in the enzymatic formation of ATP catalyzed by the pancreatic tryptophan-activating enzyme in the presence of pyrophosphate (12). However, the compounds considered to be the 2′(3′)-amino acid esters of AMP were not active when tested under the same conditions.[3]

Reaction of Amino Acyl Adenylates with Rat Liver Preparations—Incubation of amino acid-labeled C^{14}-glycyl adenylate or C^{14}-tryptophanyl adenylate with a mixture of the supernatant and microsome fractions of rat liver led to significant incorporation of isotope into the microsomal and supernatant fraction proteins subsequently isolated. As indicated in Table III, incorporation of isotope from the C^{14}-amino acyl adenylates was significantly greater than that observed in control experiments in which the amino acyl adenylate preparations were hydrolyzed before addition of the tissue preparation. The rat liver system employed here was prepared according to Zamecnik and Keller (26). In the presence of an ATP-generating system, this preparation catalyzed the incorporation of C^{14}-tryptophan and C^{14}-glycine into the microsomal proteins; the extent of the incorporation was of the same order of magnitude as that observed by these workers. It is of interest that the proteins of both the supernatant and the microsomal fraction became labeled when these were incubated with C^{14}-amino acyl adenylates. In experiments with heated rat liver preparations, the extent of incorporation into the proteins of both fractions was much greater than that observed with unheated preparations (Table III). Isolated microsomes became more extensively labeled than did microsomes in the presence of the supernatant fraction. This result may be associated with denaturation of the washed microsomal protein; furthermore, in the presence of unheated supernatant fraction there was considerable degradation of amino acyl adenylate. Experiments were also carried out with C^{14}-glycyl adenylate of very high specific activity (10^7 c.p.m. per μmole); this permitted use of relatively low concentrations of the anhydride. With concentrations of glycyl adenylate of from 10^{-3} to 10^{-6} M, the labeling of the heated protein preparations was between 5 and

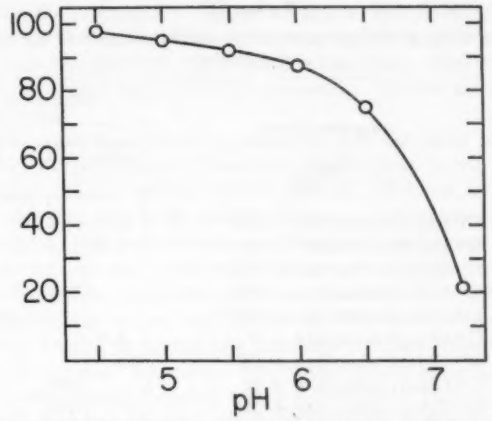

FIG. 2. Effect of pH on the hydrolysis of L-tryptophanyl adenylate. The ordinate represents percentage of initial hydroxamic acid formed. The amino acyl adenylate (1.5 μmole) was dissolved in 0.2 ml. of 0.1 M buffer (sodium acetate, pH 4.5 to 6.0; potassium phosphate, pH 6.5, 7.2); after 15 minutes at 37°, the sample was treated with 0.5 ml. of 2 M hydroxylamine hydrochloride (adjusted to pH 6.5); 5 minutes later, 1.5 ml. of ferric chloride reagent were added.

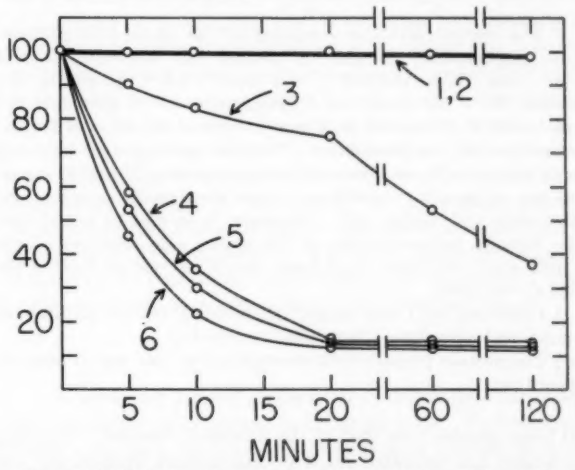

FIG. 3. Hydrolysis of several acyl adenylates. The ordinate represents percentage of initial hydroxamic acid formed. The acyl adenylates (1.0 to 2.0 μmoles) were dissolved in 0.2 ml. of 0.1 M potassium phosphate buffer (pH 7.2) and incubated at 37°; at the indicated intervals, the samples were treated with hydroxylamine and ferric chloride reagent as described in Fig. 1. *Curve 1*, carbobenzoxy-L-tryptophanyl adenylate; *Curve 2*, benzoyl adenylate; *Curve 3*, β-alanyl adenylate; *Curve 4*, glycyl adenylate; *Curve 5*, L-glutaminyl adenylate; *Curve 6*, L-tryptophanyl adenylate.

[2] Preparations of amino acyl adenylates obtained by condensing free amino acids with AMP in the presence of *N,N′*-dicyclohexylcarbodiimide (15, 21) also contained the compound that moved toward the anode at pH 5.9.

[3] Unpublished experiments.

TABLE III

Labeling of rat liver proteins by incubation with C^{14}-amino acyl adenylates

Experiment No.	Reaction mixture*	Incorporation		
		Supernatant	Microsomes	Combined
		c.p.m./mg. protein		
1	C^{14}-Glycyl adenylate + MS	11.3	5.62	
	C^{14}-Glycine + AMP + MS	0.56	0.34	
2	C^{14}-Glycyl adenylate + MS†			0.20
	C^{14}-Glycyl adenylate + MS			35.0
	C^{14}-Glycyl adenylate + MS‡			195
3	C^{14}-Glycyl adenylate + S	8.0		
	C^{14}-Glycyl adenylate + S‡	484		
	C^{14}-Glycyl adenylate + M		300	
	C^{14}-Glycyl adenylate + M‡		750	
4	C^{14}-Tryptophanyl adenylate + MS†			17.0
	C^{14}-Tryptophanyl adenylate + MS			42.0
	C^{14}-Tryptophanyl adenylate + MS‡			95.0
5	C^{14}-Tryptophanyl adenylate + MS	34	45	
	C^{14}-Tryptophanyl adenylate + S	29		
	C^{14}-Tryptophanyl adenylate + S‡	166		
	C^{14}-Tryptophanyl adenylate + M		89	
	C^{14}-Tryptophanyl adenylate + M‡		284	

* The reaction mixtures consisted of 1 ml. of rat liver preparation (supernatant (S) and microsomal (M) fractions; or both (MS); see "Experimental"), and glycyl(-1-C^{14}) adenylate (2.5 μmoles, 3.6×10^5 c.p.m.) or tryptophanyl(-3-C^{14}) adenylate (1.0 μmole, 1.9×10^5 c.p.m.) in a final volume of 1.5 ml.; pH 7.4; incubated at 38° for 30 minutes. With the use of glycine-1-C^{14} and tryptophan-3-C^{14}, we observed incorporations of 57 and 81 c.p.m. per mg. of protein, respectively, with the system described by Zamecnik and Keller (26). However, it should be noted that the specific radioactivities of the amino acid moieties of the amino acyl adenylates used here were 5 per cent of those of the free amino acids.

† Perchloric acid was added immediately before addition of amino acyl adenylate ("zero time" control).

‡ The protein preparations were heated at 100° for 10 minutes before use.

10 times greater than that of the unheated material. Labeling of heated and unheated rat liver supernatant fraction and rat liver microsomes by incubation with C^{14}-amino acyl adenylates proceeded more rapidly at pH values of 6.5 to 8.5 than at lower or higher values of pH; labeling occurred rapidly, usually reaching a maximal value within 5 minutes. With both heated and unheated preparations, the labeled amino acids were tightly bound to the proteins. The labeled amino acids were released from such preparations over a period of 32 hours by treatment with 6 N hydrochloric acid at 105°. Thus, a protein preparation labeled with C^{14}-glycyl adenylate liberated 11, 22, 28, 38, 75, 91, and 100 per cent of its total radioactivity as glycine-1-C^{14} after 1, 2, 5, 8, 12, 16, and 32 hours of hydrolysis, respectively. Treatment of the labeled heated and unheated rat liver preparations with 1-fluoro-2,4-dinitrobenzene followed by acid hydrolysis (41), gave preparations of 2,4-dinitrophenyl amino acids that contained from 70 to 80 per cent of the radioactivity originally incorporated into the proteins (Table IV). The findings strongly suggest that the labeling of proteins observed after incubation with C^{14}-amino acyl adenylates is mainly due to nonenzymatic acylation of the reactive groups of the proteins. It appears

TABLE IV

Amino end group analysis of labeled proteins

Original reaction mixture*	Incorporated isotope found as DNP-amino acid
	%
C^{14}-Glycyl adenylate + MS	74
C^{14}-Glycyl adenylate + MS†	80
C^{14}-Tryptophanyl adenylate + MS	69
C^{14}-Tryptophanyl adenylate + MS†	79
C^{14}-Tryptophanyl adenylate + MS‡	43

* The reaction mixtures contained initially 1 ml. of rat liver preparation (supernatant (S) and microsomal (M) fractions; or both (MS) and C^{14}-glycyl adenylate or C^{14}-tryptophanyl adenylate as given in Table III. The protein was subsequently precipitated and washed as described in "Experimental," and then treated with 1-fluoro-2,4-dinitrobenzene; the radioactivity of the isolated 2,4-dinitrophenyl amino acid (DNP-amino acid) fraction was determined.

† The rat liver preparation was heated at 100° for 10 minutes.

‡ A mixture consisting of 1 μmole each of the adenylates of unlabeled glycine, tyrosine, phenylalanine, isoleucine, leucine, and valine was added to the initial reaction mixture. Less isotope was also found in the DNP-amino acid fraction when unlabeled amino acyl adenylates were added after incubation of the protein preparation with C^{14}-amino acyl adenylates.

probable that such acylation involves the free amino groups of the proteins, including the ϵ-amino groups of lysine. Such an interpretation is consistent with the findings of Porter (42), who observed an increase in the number of ϵ-amino groups of proteins available for acylation after heat denaturation. Other results that are consistent with the interpretation that the observed labeling is mainly due to nonenzymatic acylation may be briefly summarized as follows. (*a*) Incubation of crystalline bovine serum albumin, ovalbumin, and ribonuclease with C^{14}-amino acyl adenylates led to considerable labeling of the proteins. (*b*) Rat liver protein preparations that were acetylated by treatment with acetic anhydride incorporated much less radioactivity than did untreated preparations when they subsequently were incubated with C^{14}-amino acyl adenylates. (*c*) Addition of ribonuclease, guanosine 5′-triphosphate, ATP, or an ATP-generating system did not influence the labeling of proteins by amino acyl adenylates. (*d*) The degree of labeling of heated proteins was generally proportional to the duration of heat treatment (0.5 to 15 minutes at temperatures of 20 to 100°). (*e*) Labeling of rat liver preparations by incubation with C^{14}-amino acyl adenylates was reduced when C^{12}-amino acyl adenylates were added to the mixture (Table IV).

Other Reactions of Amino Acyl Adenylates—The high reactivity of amino acyl adenylates is indicated not only by their rapid reaction with hydroxylamine and proteins, but also with ammonia, amino acids, AMP, and ribonucleic acid preparations. Incubation of amino acyl adenylates with concentrated ammonium hydroxide at 26° for 3 minutes gave the corresponding amino acid amides which were identified by paper chromatography; the formation of the amides of leucine, proline, alanine, glycine, tyrosine, isoleucine, and valine was observed in this manner. Similarly, incubation of amino acyl adenylates with amino acids gave the corresponding peptides. For example, incubation of glycyl adenylate (0.01 M) and L-phenylalanine (0.2

M) at pH values from 7.5 to 9.0 for 10 minutes at 26° gave glycyl phenylalanine, the formation of which was qualitatively demonstrated by paper chromatography. That amino acyl adenylates can react with the ribose hydroxyl groups of AMP is suggested by observations described above. The formation of the 2′(3′)-amino acid ester of AMP could occur by acylation of free AMP present in the preparation or possibly by an intramolecular rearrangement. However, evidence for the acylation of AMP by amino acyl adenylates has been obtained by incubation of C^{14}-AMP with tryptophanyl adenylate; significant quantities of a labeled AMP-tryptophan compound with the properties of AMP-2′(3′)-tryptophanate were formed.[3] As noted previously (1), we have also observed labeling of ribonucleic acid preparations by incubation of these with C^{14}-amino acyl adenylates. Investigation of this labeling is complicated by the usual difficulties in characterizing ribonucleic acid preparations, and also by the observation that the C^{14}-amino acid is apparently bound to ribonucleic acid by linkages of considerably different stabilities. Further work is necessary in order to determine the nature of the reaction of amino acyl adenylates with ribonucleic acid preparations.

DISCUSSION

It is of interest that some preparations of amino acyl adenylates contain isomeric compounds that appear to be the 2′(3′)-amino acid esters of AMP. The latter compounds react much more rapidly with hydroxylamine than do the corresponding amino acid ethyl esters; at least in one instance (proline), the anhydride and the isomeric ester of AMP exhibited approximately the same stability at pH 7.2 and 37° in 0.1 M potassium phosphate buffer. Further studies on these compounds are in progress. The carbobenzoxy amino acyl adenylates exhibit much less tendency to hydrolysis, and do not undergo spontaneous conversion to a ribose acylated product to a detectable extent. Formation of a ribose-acylated derivative of AMP has previously been observed by Jencks (43), who obtained adenosine 5′-phosphate 2′(3′)-acetate by the action of imidazole on acetyl adenylate. Wieland *et al.* (44) obtained a product from monosodium AMP and valylthiophenol hydrochloride which is considered (45) to be the 2′(3′)-valine ester of AMP. Weiss *et al.* (13) have observed formation of a compound exhibiting the properties of adenosine 5′-triphosphate 2′(3′)-tryptophanate in the tryptophan-activating system. Studies on the enzymatic behavior of the amino acid esters of nucleotides may be of interest, especially in the light of recent evidence that amino acids are enzymatically incorporated into ribonucleic acid by ester linkages involving the hydroxyl groups of the terminal adenosine moieties (46). As stated above, preliminary experiments indicate that the amino acid esters of AMP are not active in the enzymatic formation of ATP catalyzed by the tryptophan-activating enzyme; however, in view of the apparent reactivity of these compounds, the possibility that they may participate in other reactions must be considered. The inactivity of the amino acid esters of AMP in ATP synthesis contrasts with the reactivity of the soluble ribonucleic acid-amino acid complexes in ATP formation.

Studies on the preparation of other amino acyl adenylates are in progress. Thus far, it would appear that the present method cannot be applied without modification to the preparation of the adenylates of cystine, lysine, and glutamic acid. Very recently, the preparation of glycyl adenylate, and DL-leucyl adenylate via the corresponding benzyl mercaptoformyl derivatives has been reported (47). It is possible that this or other blocking techniques may be useful in the preparation of other amino acyl adenylates.

The available evidence is consistent with the belief that the labeling of proteins by C^{14}-amino acyl adenylates in our studies is largely due to nonenzymatic acylation. However, it is conceivable that some of the observed incorporation is enzymatically catalyzed, and that extensive nonenzymatic acylation by the relatively high concentrations of amino acyl adenylate employed has masked an enzymatic reaction. Zioudrou *et al.* (23) have independently carried out similar studies; these workers observed labeling of heated and unheated rat liver mitochondria by C^{14}-tyrosinyl adenylate, C^{14}-glycyltyrosinyl adenylate, and the corresponding *N*-carbobenzoxy derivatives. They have concluded that the labeling of the mitochondria is due to nonenzymatic acylation. Other studies in our laboratory indicate that enzymatically-synthesized tryptophanyl adenylate can acylate rat liver microsome preparations, other proteins (*e.g.* crystalline bovine serum albumin, ovalbumin), and the tryptophan-activating enzyme itself.[3] It is therefore possible that some of the labeling of proteins observed when C^{14}-amino acids are incubated with isolated preparations and an ATP-generating system may be due to this type of acylation and therefore be artifactitious. The present findings re-emphasize the need for studies in which a specific protein is isolated, and in which the incorporated amino acid is shown to be in positions corresponding to those of the same amino acid in the native protein. It is evident that the high reactivity of the amino acyl adenylates poses difficult problems not only in the preparation of these compounds, but also in their study in biochemical systems. Nevertheless, the ability of amino acyl adenylates to react as substrates in ATP synthesis by reversal of Reaction 1 suggests that it may be possible to design systems for the study of other enzymatic reactions in which these anhydrides participate.

SUMMARY

1. A procedure for the preparation of amino acyl adenylates has been devised and applied to 15 amino acids. Carbobenzoxy amino acids were condensed with adenosine 5′-phosphate in the presence of *N*,*N*′-dicyclohexylcarbodiimide; the *N*-carbobenzoxy amino acyl adenylates were isolated and converted to the corresponding amino acyl adenylates by catalytic hydrogenation.

2. When glycyl adenylate and tryptophanyl adenylate labeled with C^{14} in the amino acid moieties were incubated with rat liver preparations capable of catalyzing the incorporation of free amino acids into protein in the presence of an adenosine triphosphate-generating system, isotope was incorporated into the proteins subsequently isolated. Liver preparations that were denatured by heating were labeled to a much greater extent by incubation with C^{14}-amino acyl adenylates. Evidence was obtained indicating that the incorporation of isotope from C^{14}-amino acyl adenylates into proteins is mainly due to nonenzymatic acylation of the proteins.

3. Spontaneous conversion of α-amino acyl adenylates to products that appear to be the corresponding isomeric adenosine 5′-phosphate-2′(3′)-amino acid esters has been observed. Certain other properties of amino acyl adenylates and the corre-

sponding *N*-carbobenzoxy amino acyl adenylates have been studied.

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REFERENCES

1. Castelfranco, P., Moldave, K., and Meister, A., *J. Am. Chem. Soc.*, **80**, 2335 (1958).
2. Berg, P., *J. Am. Chem. Soc.* **77**, 3163 (1955).
3. Berg, P., *J. Biol. Chem.*, **222**, 1025 (1956).
4. Hoagland, M. B., *Biochim. et Biophys. Acta*, **16**, 288 (1955).
5. Hoagland, M. B., Keller, E. B., and Zamecnik, P. C., *J. Biol. Chem.*, **218**, 345 (1956).
6. Davie, E. W., Koningsberger, V. V., and Lipmann, F., *Arch. Biochem. Biophys.*, **65**, 21 (1956).
7. DeMoss, J. A., and Novelli, G. D., *Biochim. et Biophys. Acta*, **22**, 49 (1956).
8. Hoagland, M. B., Zamecnik, P. C., Sharon, N., Lipmann, F., Stulberg, M. P., and Boyer, P. D., *Biochim. et Biophys. Acta*, **26**, 215 (1957).
9. Bernlohr, R., and Webster, G. C., *Arch. Biochem. Biophys.*, **73**, 276 (1958).
10. Novelli, G. D., *Proc. Natl. Acad. Sci. U. S.*, **44**, 86 (1958).
11. Berg, P., *J. Biol. Chem.*, **233**, 601 (1958).
12. Karasek, M., Castelfranco, P., Krishnaswamy, P. R., and Meister, A., *J. Am. Chem. Soc.*, **80**, 2335 (1958).
13. Weiss, S. B., Acs, G., and Lipmann, F., *Federation Proc.*, **17**, 333 (1958).
14. Zamecnik, P. C., Stephenson, M. L., and Hecht, L. I., *Proc. Natl. Acad. Sci. U. S.*, **44**, 73 (1958).
15. Kingdon, H. S., Webster, L. T., Jr., and Davie, E. W., *Proc. Natl. Acad. Sci. U. S.*, **44**, 757 (1958).
16. Jencks, W. P., and Lipmann, F., *J. Biol. Chem.*, **225**, 207 (1957).
17. Moyed, H. S., and Lipmann, F., *J. Bacteriol.*, **73**, 117 (1957).
18. Moldave, K., and Meister, A., *J. Biol. Chem.*, **229**, 463 (1957).
19. Kellerman, G. M., *J. Biol. Chem.*, **231**, 427 (1958).
20. DeMoss, J. A., Genuth, S. M., and Novelli, G. D., *Proc. Natl. Acad. Sci. U. S.*, **42**, 325 (1956).
21. Berg, P., *J. Biol. Chem.*, **233**, 608 (1958).
22. Khorana, H. G., and Todd, A. R., *J. Chem. Soc.*, 2257 (1953).
23. Zioudrou, C., Fujii, S., and Fruton, J. S., *Proc. Natl. Acad. Sci. U. S.*, **44**, 439 (1958).
24. Bergmann, M., and Zervas, L., *Chem. Ber.*, **67**, 1192 (1932).
25. Renfrow, W. B., Jr., and Hauser, C. P., *J. Am. Chem. Soc.*, **59**, 2308 (1937).
26. Zamecnik, P. C., and Keller, E. B., *J. Biol. Chem.*, **209**, 337 (1954).
27. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950).
28. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **195**, 257 (1952).
29. Tyner, E. P., Heidelberger, C., and LePage, G. A., *Cancer Research*, **13**, 186 (1953).
30. Moldave, K., *J. Biol. Chem.*, **221**, 543 (1956).
31. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
32. Markham, R., and Smith, J. B., *Biochem. J.*, **52**, 552 (1952).
33. Nikiforuk, G., and Colowick, S. P., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. II*, Academic Press, Inc., New York, 1955, p. 469.
34. Schmidt G., Cubiles, R., Zöllner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M., and Thannhauser, S. J., *J. Biol. Chem.*, **192**, 715 (1951).
35. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211**, 907 (1954).
36. Troll, W., and Lindsley, J., *J. Biol. Chem.*, **215**, 655 (1955).
37. Meister, A., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **192**, 535 (1951).
38. Jencks, W. P., *Biochim. et Biophys. Acta*, **27**, 417 (1958).
39. Raacke, I. D., *Biochim. et Biophys. Acta*, **27**, 416 (1958).
40. Goldenberg, V., and Spoerri, P., *Anal. Chem.*, **30**, 1327 (1958).
41. Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., *Methods of Biochem. Anal.*, **2**, 359 (1955).
42. Porter, R. R., *Biochim. et Biophys. Acta*, **2**, 105 (1948).
43. Jencks, W. P., *Biochim. et Biophys. Acta*, **24**, 227 (1957).
44. Wieland, T., Niemann, E., and Pfleiderer, G., *Angew. Chem.*, **68**, 305 (1956).
45. Wieland, T., and Pfleiderer, G., *Advances in Enzymol.*, **19**, 235 (1957).
46. Zachau, H. G., Acs, G., and Lipmann, F., *Proc. Natl. Acad. Sci. U. S.*, **44**, 885 (1958).
47. McCorquodale, D. J., and Mueller, G. C., *Arch. Biochem. Biophys.*, **77**, 13 (1958).

The Enzymatic Exchange of L-Serine with *O*-Phospho-L-serine Catalyzed by a Specific Phosphatase*

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During the course of experiments on the enzymatic synthesis of phosphatidylserine it was found that extracts of rat liver catalyzed the following reactions.

$$\text{P-L-serine}^1 \rightarrow \text{L-serine} + P_i \quad (1)$$

$$\text{P-D-serine} \rightarrow \text{D-serine} + P_i \quad (2)$$

$$\text{P-L-serine} + \text{L-serine-3-C}^{14} \rightleftharpoons \text{P-L-serine-3-C}^{14} + \text{L-serine} \quad (3)$$

An enzyme from chicken liver catalyzing the same reactions was discovered independently by Neuhaus and Byrne (1).

This paper will describe the partial purification and some of the properties of the enzyme from rat liver. A preliminary report on some aspects of this work has been published (2).

EXPERIMENTAL

Materials and Methods—DL-Serine-3-C^{14} was purchased from the Volk Radiochemical Company. D-Serine-3-C^{14} and L-serine-3-C^{14} were generous gifts of Dr. Manfred Karnovsky.

Orthophosphate labeled with P^{32} was obtained from the Atomic Energy Commission. ATP labeled in the two terminal phosphates with P^{32} was prepared by a method similar to that of Lowenstein (3).

P_i was determined by a procedure based on that of Gomori (4). Protein was determined by the method of Warburg and Christian (5). P-Serine and serine were separated by chromatography on Whatman No. 43 filter paper with the ascending technique. The solvent system was 70 per cent ethanol containing 0.02 M citrate buffer of pH 4.8. The R_F value for serine in this system was 0.55 and for P-serine, 0.27.

Synthesis of P^{32}-Serine—Neuhaus and Korkes (6) have described a convenient procedure for the preparation of P-serine in unlabeled form. However, it is not easy to adapt this procedure for the preparation of P^{32}-labeled serine. Attempts to use the procedure of Plimmer (7) for this synthesis were unsuccessful, but the following modification of the method was found satisfactory.

Carrier-free P^{32}, 1 mc., was added to a test tube containing 0.2 ml. of 85 per cent orthophosphoric acid. The solution was kept in an oven at 110° overnight. After the tube was cooled, 0.4 gm. of phosphoric anhydride was cautiously added, followed by 0.2 gm. of L-serine. The mixture was stirred until it formed a sticky paste. The tube was then tightly stoppered and set aside for 3 days at 45°. Water, (2.5 ml.), was then added, and the solution was heated in a boiling water bath for 15 minutes. This treatment hydrolyzes undesired pyrophosphates and diesters.

The solution was diluted to about 200 ml. with water and neutralized with hot aqueous barium hydroxide, and then was chilled in an icebox overnight. The precipitate was removed by centrifugation and washed several times with cold water. The combined supernatant solution and washings were then passed over a column of Dowex 1-formate, 19 cm. long and containing 9 ml. of packed resin. The column was then washed with several bed volumes of water and eluted with a gradient elution technique. The upper reservoir contained 0.5 M formic acid and the mixing chamber (300 ml.) distilled water initially. Three fractions of 14 ml. each were collected per hour. The P-serine appeared in a band collected between 240 to 288 ml. The fractions containing P-serine were combined and evaporated to 2 ml. The solution was chilled to 0°, and 3 volumes of ethanol were added slowly with good stirring. The solution was then held for 24 hours in an icebox. The crystalline precipitate was removed by centrifugation and washed twice with 1.5 ml. of cold ethanol and then dried in a vacuum desiccator. The dried product weighed 100 mg. (29 per cent yield based on serine) and melted with decomposition between 166 to 168 (uncorrected).

P-L-Threonine and P-L-tyrosine were prepared by the original method of Plimmer.

Preparation of Enzyme—Adult female albino rats were killed by decapitation, and the livers were immediately removed and homogenized in 9 volumes of ice-cold Tris buffer of pH 7.4, with the use of a Waring Blendor for 1 minute. All subsequent operations were carried out at 0° unless otherwise specified.

Step 1. Precipitation at pH 5.2—The pH of the homogenate was adjusted to 5.2 by the dropwise addition of 1 M acetic acid. The copious precipitate was removed by centrifugation in a refrigerated Servall centrifuge and discarded.

Step 2. Heat Treatment—The supernatant solution was adjusted to pH 6.4 by addition of 0.5 Tris buffer of pH 8.8, and $MgCl_2$ was added to a final concentration of 0.015 M. The temperature of the enzyme preparation was then rapidly raised to 60° by immersion in a hot water bath with efficient stirring and maintained at 60° for 40 minutes. The suspension was chilled to room temperature, and the precipitate was removed by centrifugation and discarded.

Step 3. Fractionation on Calcium Phosphate Gel—The pro-

* Supported by grants from the Nutrition Foundation, the Life Insurance Medical Research Fund, and the National Institute of Neurological Diseases and Blindness, United States Public Health Service (Grant B-1199).

[1] The abbreviations used are: P-L-serine, *O*-phospho-L-serine; P-D-serine, *O*-phospho-D-serine; P_i, orthophosphate; Tris, tris-(hydroxymethyl)aminomethane.

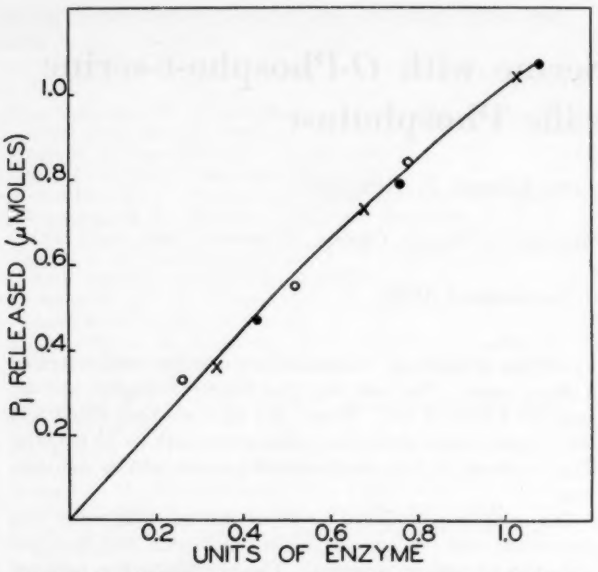

FIG. 1. P_i release with varying amounts of enzyme. Each tube contained 4 μmoles of P-D-serine, 20 μmoles of Tris buffer of pH 7.4, 16 μmoles of $MgCl_2$, and varying amounts of enzyme in a final volume of 1.0 ml. The incubation was for 1 hour at 37° after which the reaction was stopped by the addition of 5 ml. of 5 per cent trichloroacetic acid. Orthophosphate determinations were done on aliquots after filtration.

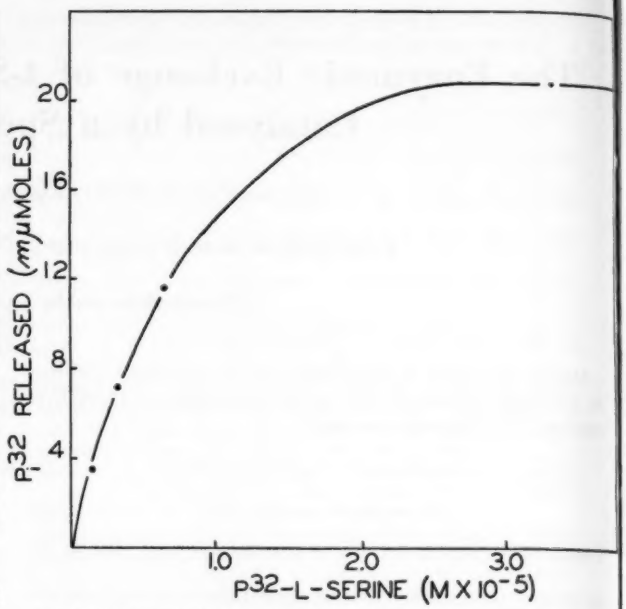

FIG. 2. Hydrolysis as a function of P^{32}-L-serine concentration. Each tube contained 16 μmoles of $MgCl_2$, 20 μmoles of Tris buffer of pH 7.4, 0.10 mg. of enzyme, and varying amounts of P^{32}-L-serine in a final volume of 3.0 ml. The tubes were incubated for 20 minutes at 37°, and the reaction was stopped by the addition of 0.4 ml. of 10 N H_2SO_4, followed by 0.02 ml. of 0.01 M K_2HPO_4 to provide carrier P_i and 0.4 ml. of 10 per cent ammonium molybdate. 5 ml. of a mixture of equal volumes of isobutanol and benzene were then added, the tubes were stoppered and inverted 100 times. After light centrifugation, 3-ml. aliquots of the organic phase were dried in aluminum cups and counted.

TABLE I

Hydrolysis of O-phospho-L-serine and O-phospho-D-serine

Each tube contained 16 μmoles of $MgCl_2$, 20 μmoles of Tris, pH 7.5, and 0.8 mg. of enzyme purified by Steps 1 and 3 only. The final volume was 1.0 ml. and the incubation was for 1 hour at 37°.

Substrates added	P_i released
	μmoles
3 μmoles P-L-serine	0.32
3 μmoles P-L-serine + 1 μmole L-serine	0.14
3 μmoles P-L-serine + 1 μmole D-serine	0.32
3 μmoles P-D-serine	0.45
3 μmoles P-D-serine + 1 μmole L-serine	0.15
3 μmoles P-D-serine + 1 μmole D-serine	0.46

tein concentration of the supernatant solution from Step 2 was usually about 4 mg. per ml. The supernatant solution was chilled to 0° and treated with one-fifth of its volume of a suspension of calcium phosphate gel prepared by the method of Tsuboi and Hudson (8). The gel was removed by centrifugation and washed successively three times each with water, 0.1 M ammonium sulfate, and 0.5 M ammonium sulfate. Usually the greatest recovery and highest specific activity was obtained in the 0.5 M ammonium sulfate extracts. The active extracts were finally dialyzed against 0.02 M Tris buffer of pH 7.4 containing 0.005 M $MgCl_2$.

Preparations of enzyme with an activity of 3 to 4 units per mg. of protein (Fig. 1) could be obtained by this procedure, representing an overall purification of 25- to 40-fold over the original homogenate with a recovery of about 15 to 25 per cent of the total activity. Although the enzyme was not pure at this point, the rather severe heat treatment eliminated many interfering enzyme activities, *e.g.* other phosphatases attacking substrates such as β-glycerophosphate and AMP.

Enzymatic Hydrolysis of P-L-Serine and P-D-Serine—An experiment illustrating the enzymatic hydrolysis of P-L-serine and P-D-serine is shown in Table I. At the concentrations shown, both substrates are readily hydrolyzed, and the reaction is strikingly inhibited by free L-serine, but not D-serine. This inhibition by L-serine appears to be characteristic of the specific P-serine phosphatase; other phosphatases present in crude liver extracts, such as those hydrolyzing β-glycerophosphate and AMP, are not inhibited by L-serine.

Since free L-serine is inhibitory to the hydrolytic reaction, P-D-serine was used as substrate in routine experiments in which hydrolysis was measured. The relation between enzyme concentration and hydrolysis is shown in Fig. 1. Since this relationship is not linear over a wide range, 1 unit of enzyme activity was defined as an amount of enzyme which liberated 1 μmole of P_i under the conditions described in Fig. 1, and lower levels of activity were read from the standard curve.

Affinity for P-D-Serine and P-L-Serine—Substrates labeled with P^{32} were used to study the affinity of the enzyme for P-L-serine and P-D-serine (Figs. 2 and 3). The procedure, described under Fig. 2, was based on that of Ernster *et al.* (9) and proved very useful for accurate estimation of the release of amounts of P_i much too low for colorimetric determination.

K_s for P-L-serine was found to be 5×10^{-6} M, calculated by

the method of Lineweaver and Burk (10), whereas that for P-D-serine was 5×10^{-3} M.

That both P-D-serine and P-L-serine are hydrolyzed at the same active site on the enzyme is indicated by the experiment shown in Table II. P^{32}-D-serine was incubated in the presence and in the absence of unlabeled P-L-serine, and the radioactivity of the P_i released was measured. The unlabeled P-L-serine effectively displaced the P^{32}-D-serine from the enzyme, as would be expected from the relative Michaelis constants.

Effect of Varying L-Serine Concentration—The inhibition of hydrolysis of P^{32}-L-serine by varying amounts of free L-serine is shown in Fig. 4. The hydrolysis is inhibited 50 per cent at about 4×10^{-4} M serine.

Specificity of Enzyme for Hydrolysis—The purified enzyme is quite specific for the hydrolysis of P-L-serine and P-D-serine (Table III). Enzymes present in the crude extracts which hydrolyze β-glycerophosphate and AMP are almost completely removed. *O*-phospho-L-threonine is only slightly hydrolyzed; the hydrolysis of *O*-phospho-L-tyrosine is apparently catalyzed by a contaminating enzyme, since it was not inhibited by L-serine, in other experiments not shown.

Requirement for Magnesium—Purified preparations of the enzyme from rat liver require added magnesium ions for activity with 2×10^{-4} M $MgCl_2$ giving half the optimal stimulation. Manganese or calcium ions were not effective in replacing magnesium (Table IV).

Heat Stability—Heating the enzyme to 60° or higher for periods of 10 minutes or more leads to complete inactivation. However, the enzyme is markedly stabilized by the presence of magnesium ions. Heating at 60° for 40 minutes in 0.015 M $MgCl_2$ usually led to loss of only 10 to 40 per cent of activity. As mentioned above, this stability to heating in the presence of magnesium proved of considerable use in ridding the enzyme of other phosphatases.

Exchange of Labeled L-Serine with P-Serine—In attempts to demonstrate a serine kinase in mammalian liver, DL-serine-3-C^{14} was incubated with ATP, $MgCl_2$, and carrier P-L-serine, with whole homogenates of rat liver used as enzyme source. Fractionation of the reaction products on Dowex-1 revealed that about one-third of the labeled serine has been converted to P-serine. Further investigation showed that the reaction was catalyzed by a soluble enzyme and that ATP was not involved. ATP^{32} labeled in the terminal phosphates did not form P^{32}-serine. Fractionation of the extracts revealed that the exchange reaction was associated with the hydrolysis of P-serine by a specific phosphatase.

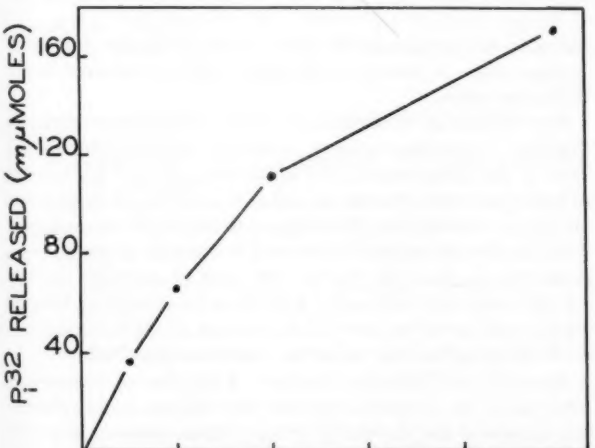

FIG. 3. Hydrolysis as a function of concentration of P^{32}-D-serine. The experimental procedures were similar to those described for Fig. 2.

TABLE II

Competition of O-phospho-D-serine and O-phospho-L-serine

Conditions of incubation and determination of the radioactivity of the P_i released were similar to those of Fig. 2. The specific activity of the P^{32}-D-serine was 104,000 counts per μmole and that of P^{32}-L-serine was 89,000. The final volume was 1.0 ml. in Experiment 1 and 3.0 ml. in Experiment 2.

Substrates added	Radioactivity released as P_i
	c.p.m.
Experiment 1	
2 μmoles P^{32}-D-serine	8265
2 μmoles P^{32}-D-serine + 2 μmoles P-L-serine	275
Experiment 2	
2 μmoles P^{32}-L-serine	3303
2 μmoles P^{32}-L-serine + 2 μmoles P-D-serine	3385

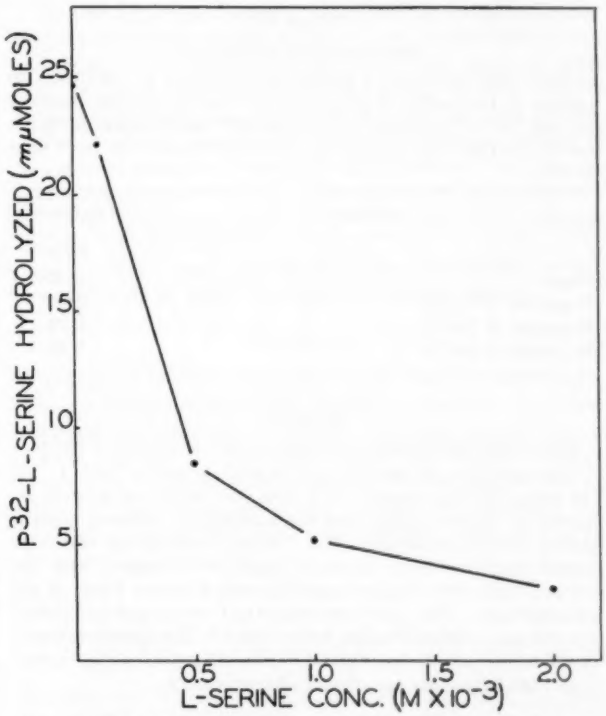

FIG. 4. Inhibition of hydrolysis of P-L-serine at varying concentrations of L-serine. Conditions of the experiment and determination of radioactive P_i released were similar to Fig. 2, except that each tube contained 2 μmoles of P^{32}-L-serine (86,000 counts per μmole) and varying amounts of L-serine as shown in a final volume of 1.0 ml.

TABLE III

Specificity of phosphoserine phosphatase

Each tube contained 20 μmoles of Tris buffer of pH 7.4, 16 μmoles of $MgCl_2$, 0.4 mg. of enzyme, and 4 μmoles of substrate as shown. The incubation was for 1 hour at 37°. The final volume was 1.0 ml.

Substrate	P_i released
Experiment 1	
P-D-serine	1.37
β-glycerophosphate	0.05
AMP	0.00
Experiment 2	
P-D-serine	0.65
P-L-tyrosine	0.28
P-L-threonine	0.07

TABLE IV

Requirement for magnesium

Each tube contained 4 μmoles of P-D-serine as substrate, 20 μmoles of Tris buffer of pH 7.4, and 0.4 ml. of enzyme dialyzed against 0.02 M Tris buffer containing 0.001 ethylenediaminetetraacetate to free it of magnesium. The incubation was for 1 hour at 37°.

Additions	P_i released
	μmoles
None	0.07
20 μmoles of $MgCl_2$	0.47
20 μmoles of $MnCl_2$	0.03
20 μmoles of $CaCl_2$	0.05

TABLE V

Enzymatic equilibration of L-serine with O-phospho-L-serine

The conditions of incubation were as described in Table I. At the end of the experiment, each tube was boiled, the protein removed by centrifugation, and the supernatant solution concentrated under a jet of air. The P-serine fraction was then separated from free serine by chromatography on paper, with the use of 70 per cent ethanol containing 0.02 M citrate buffer of pH 4.8 as solvent. The spots containing the P-serine and serine were cut out and counted in a gas flow counter. The specific activity of the labeled serine was 135,000 counts per μmole in Experiment 1, and 131,000 counts per μmole in Experiment 2.

Substrates added	Percentage of radioactivity converted to P-serine
Experiment 1	
5 μmoles P-L-serine + 1 μmole L-serine-3-C^{14}	31.5
5 μmoles of P-L-serine + 1 μmole D-serine-3-C^{14}	1.7
Experiment 2	
2 μmoles P-L-serine + 1 μmole DL-serine-3-C^{14}	19.1
2 μmoles P-D-serine + 1 μmole DL-serine-3-C^{14}	0.61

The experiment shown in Table V indicates that free L-serine exchanges with P-L-serine under conditions in which free D-serine does not. Also, free L-serine exchanges with P-L-serine, but not with P-D-serine. In view of the evidence that P-D-serine uses

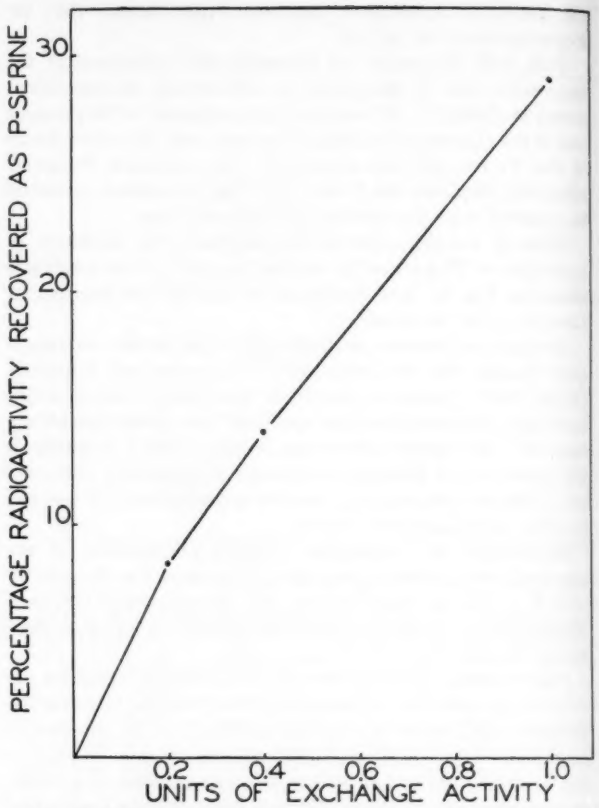

FIG. 5. Conversion of L-serine-3-C^{14} to P-L-serine as a function of enzyme concentration. Each tube contained 5 μmoles of P-L-serine, 0.50 μmole of L-serine-3-C^{14} (130,000 counts per μmole), 15 μmoles of $MgCl_2$, 20 μmoles of Tris buffer of pH 7.4, and varying amounts of enzyme as shown in a final volume of 1.0 ml. The incubation was for 1 hour at 37°. The percentage of radioactivity recovered as P-serine was estimated as in the experiment described in Table V.

the same site on the enzyme as P-L-serine (Table II), this result is surprising. A possible explanation will be considered in the discussion below.

The percentage of radioactive serine which is converted to P-serine is dependent upon a number of factors including the ratio of the concentration of P-serine to serine and the amount of hydrolysis of the P-serine as well as the activity of the enzyme. However, conditions can be arranged in which the percentage of radioactivity converted to P-serine is a function of enzyme concentration as shown in Fig. 5. The unit of exchange activity for this enzyme is arbitrarily defined as an amount of enzyme which leads to the recovery of 29 per cent of the total radioactivity as phosphoserine under the conditions described.

Specificity of Exchange Reaction—A number of compounds were tested to determine whether the enzyme could catalyze the transfer of the phosphoryl group of these compounds to free labeled serine. These phosphorylated compounds included the following: phosphorylcholine, phosphorylethanolamine, phosphocreatine, ATP, UTP, CTP, GTP, α-glycerophosphate, β-glycerophosphate, fructose 1,6-diphosphate, glucose 6-phosphate, and orthophosphate. None of these compounds could replace P-L-serine in the conversion of free labeled serine to

P-serine. A number of other compounds were tested to see whether the phosphate group of P-L-serine could be transferred to them, by incubating the suspected compounds with P-L-serine and labeled serine. If the compound were active, it should compete with the labeled serine and thus conversion of labeled serine to P-serine should be diminished. No such activity was detected with the following compounds: choline, ethanolamine, glycerol, glucose, threonine, creatine, fructose, or dephosphorylated phosvitin. This assay for phosphate acceptor compounds is not as sensitive as the assay for phosphate donors.

Evidence that Hydrolysis and Exchange are Catalyzed by Same Enzyme—The following experimental observations support the conclusion that hydrolysis and exchange are catalyzed by the same enzyme. (*a*) The ratio of the two activities remains constant during fractionation. The ratio of hydrolysis activity to exchange activity was 1.5. (*b*) Both activities display the same heat stability. (*c*) Both activities require magnesium ions. (*d*) Both exhibit the same high substrate specificity, except that P-D-serine is an effective substrate for hydrolysis, but not for exchange. This point will be considered in the discussion.

Other Properties of Enzyme—In their study of the closely similar or identical enzyme from chicken liver, Neuhaus and Byrne (1) have established that the inhibition of the hydrolysis of P-serine by L-serine is not competitive and have shown that the pH optimum for exchange and hydrolysis is different. These points have not been further investigated in the present study.

DISCUSSION

The enzymatic reactions described are consistent with the following scheme for the mechanism of this specific phosphatase.

$$\text{P-L-Serine} + \text{enzyme} \rightleftharpoons \text{enzyme-P} + \text{L-serine}$$

$$\text{Enzyme-P} + H_2O \rightarrow \text{enzyme} + P_i$$

At first it was thought that the failure of P-D-serine to act as a phosphate donor, although readily hydrolyzed at the same enzymatic site as P-L-serine, necessitated a more complex mechanism. The probable explanation of this fact, first suggested by Neuhaus,[2] is that L-serine is, in fact, converted to P-L-serine during the hydrolysis of P-D-serine, but since the affinity of P-L-serine for the enzyme is about 1000 times greater than that of P-D-serine, the newly formed P-L-serine is at once re-hydrolyzed. In other words, the pool of P-D-serine cannot act as a trap for P-L-serine. This explanation requires that if P^{32}-D-serine and a pool of unlabeled P-L-serine are incubated in the presence of L-serine, a slow transfer of radioactivity to P-L-serine should take place. This experiment, although feasible, is technically difficult.

The failure of free D-serine either to inhibit the hydrolysis or participate in the exchange reaction is probably due to the much lower affinity of D-serine than L-serine.

Examples of phosphatases which also carry out transfer or exchange reactions are now well known, but the exchange reaction described here has several novel aspects. Usually, rapid exchange or transfer is catalyzed only by nonspecific phosphatases (11, 12), whereas the phosphoserine phosphatase is highly specific. Furthermore, usually a very high concentration of acceptor is needed to compete with water in the hydrolysis, whereas low concentrations of L-serine are quite effective here.

Koshland (13) has discussed the criteria for deciding whether a given enzymatic reaction proceeds by a double displacement mechanism. The rate of exchange and the high degree of specificity of the P-serine phosphatase meet these criteria, offering support but not conclusive proof for the postulated mechanism.

The possibility was considered that the hydrolytic actions of the enzyme might be, in a sense, an artifact and that the true function of the enzyme *in vivo* might be to carry out the transfer of phosphate to some other acceptor, but no such compound has as yet been found.

In our experiments, as in those of other laboratories,[2] no evidence for a serine kinase utilizing ATP could be found.

SUMMARY

This paper describes the partial purification and some properties of a specific phosphoserine phosphatase from rat liver which catalyzes the hydrolysis of phospho-L- or phospho-D-serine and a rapid exchange of labeled free L-serine with phospho-L-serine. The enzyme is highly specific and requires magnesium ions for activity. The mechanism of the reaction, which probably proceeds by a double displacement with a phosphorylated enzyme intermediate, is discussed.

REFERENCES

1. Neuhaus, F., and Byrne, W. L., *Biochim. et Biophys. Acta*, **28**, 223 (1958).
2. Borkenhagen, L. F., and Kennedy, E. P., *Biochim. et Biophys. Acta*, **28**, 222 (1958).
3. Lowenstein, J. M., *Biochem. J.*, **65**, 197 (1957).
4. Gomori, G., *J. Lab. Clin. Med.*, **27**, 955 (1942).
5. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941).
6. Neuhaus, F., and Korkes, S., *Biochem. Preparations*, **6**, 75 (1958).
7. Plimmer, R. H. A., *Biochem. J.*, **35**, 462 (1941).
8. Tsuboi, K. K., and Hudson, P. B., *J. Biol. Chem.*, **224**, 879 (1957).
9. Ernster, L., Zetterström, R., and Lindberg, O., *Anal. Chem.*, **4**, 942 (1950).
10. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
11. Morton, R. K., in W. D. McElroy, and H. B. Glass (Editors), *Phosphorus metabolism, Vol. II*, Johns Hopkins Press, Baltimore, 1952, p. 125.
12. Morton, P. K., *Discussions Faraday Soc.*, **20**, 149 (1955).
13. Koshland, D. E., Jr., *Discussions Faraday Soc.*, **20**, 142 (1955).

[2] Personal communication from Drs. F. Neuhaus and W. L. Byrne.

Chromatographic Evidence on the Occurrence of Thiotaurine in the Urine of Rats Fed with Cystine*

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Rats fed a diet supplemented with L-cystine excrete in the urine taurine, hypotaurine (1–3), and possibly cystamine disulfoxide (4), although the excretion of the last mentioned compound is not certain (3). It was recognized earlier (1) that paper chromatograms of the urine of these rats showed another ninhydrin-positive spot which moved more rapidly than did taurine in collidine-lutidine solvent. The nature of the new compound was not investigated because it did not appear in all chromatograms and because it represented only a small fraction of the sulfur compounds excreted. More recently, an unknown cystine metabolite has been observed by chromatography by Awapara in the organs of rats treated with injections of S^{35}-cystine (5). We have now accumulated evidence for the identification of the new compound as the thiosulfonate analogue of taurine, aminoethylthiosulfonate ($NH_2—CH_2—CH_2—SO_2—SH$). Following Sörbo (6), the term thiotaurine will be retained for this compound.

EXPERIMENTAL

The following compounds were prepared in this laboratory: cysteine sulfinic acid (7), cysteic acid (8), cysteinesulfonate (9), alaninethiosulfonate (10), cystine disulfoxide (11), cystamine disulfoxide (12), hypotaurine (13), lanthionine (14, 15), lanthionine sulfoxide (15), lanthionine sulfone (16), lanthionamine (17), lanthionamine sulfoxide (17). Cysteaminesulfonate was prepared by treatment of cystamine solutions with excess sodium bisulfite before the chromatographic run. The synthesis of thiotaurine will be described in the text. The remaining products were obtained commercially.

Paper chromatography was performed using Whatman No. 4 paper. The solvents were phenol saturated with water and a mixture of equal volumes of collidine and lutidine, saturated with 1 volume of water. In the two-dimensional chromatography the phenol solvent was used first.

Male rats of the Wistar strain weighing 200 to 300 gm. were placed in pairs in metabolic cages and given the basal diet previously described (18). When desired, the animals were given a diet of the same composition supplemented with 6 or 12 per cent of L-cystine. The urine was collected over 2 ml. of 2 N HCl every 24 hours and diluted to 40 ml. with water.

Urinary thiosulfonate was determined by cyanolysis as described by Sörbo (6). This reaction did not give values that were proportional to the amount of urine tested. Better proportionality was obtained by using twice the amount of the reactants employed by Sörbo and a sample of urine not exceeding $\frac{1}{40}$ of the 24-hour sample. Nevertheless, the results obtained when this method was applied to urine were only semiquantitative.

RESULTS AND DISCUSSION

Detection of New Compound on Paper Chromatograms—The purpose of the first part of this work was to obtain the reproduceable appearance of the new spot on the chromatograms of the urine of rats. It was soon recognized that this spot was invariably present on the chromatograms when $\frac{1}{100}$ of the 24-hour urine specimen of two rats receiving the diet containing 6 per cent cystine was spotted on the paper. The compound appears as a ninhydrin-positive elongated spot above the position of taurine. Although the position of this compound in the chromatogram is definite, with this relatively large sample of urine the chromatograms are distorted and somewhat confused, possibly because of the effects of large amounts of taurine, salts, and other compounds. When the dietary cystine was increased from 6 to 12 per cent, the spot was easily detected with $\frac{1}{200}$ and even with $\frac{1}{400}$ of the 24-hour sample of urine. Accordingly, most of the work was performed using the higher level of dietary cystine.

Fig. 1 illustrates a typical chromatogram made with $\frac{1}{200}$ of of the 24-hour urine sample of two rats on the diet containing 12 per cent cystine. The spot marked 3 is the one under study in the present paper; it will be referred to as compound 3 in this paper. Unless the rats were given extra cystine this spot was not seen, even when more urine was used for chromatography.

Elution of Compound 3 and its Conversion to Taurine—The area presumed to contain compound 3 was cut from 6 chromatograms similar to that illustrated, and the remaining paper was developed with ninhydrin in order to ascertain that most of the compound was removed and that it was not contaminated with taurine. All of the paper fragments were eluted with water, the eluates were pooled, and the resulting solution was divided into two portions. The first was used as a control and was rechromatographed without further treatment. The second was treated with 1 ml. of 30 per cent hydrogen peroxide in the presence of a trace of ammonium molybdate (Dent (19)) and then rechromatographed. The first chromatogram showed compound 3 unchanged, whereas the second showed only taurine. This result suggests that compound 3 is a sulfur derivative of ethylamine which is not identical with taurine.

Cysteamine, cystamine, cystamine disulfoxide, and hypotaurine are all sulfur derivatives of ethylamine easily converted

* This work has been assisted by a grant of the Rockefeller Foundation to the laboratory. It forms a part of a research program on the biochemistry of sulfur compounds sponsored by the Consiglio Nazionale Ricerche Nucleari.

to taurine by hydrogen peroxide; nevertheless, none of these possesses the chromatographic coordinates of compound 3, a fact that suggests that the sulfur-containing moiety of compound 3 has an unusual structure.

Determination of Oxidation Level of Sulfur Compounds on Paper Chromatograms—Procedures for the detection of sulfur-containing compounds on paper chromatograms have occasionally been reported in the literature. By appropriate use of these procedures, together with another devised for that purpose in the present paper, it is possible to establish the degree of oxidation of the sulfur group of an organic sulfur-containing compound found on a paper chromatogram. A summary of these reactions as applied to a group of representative sulfur compounds is seen in Table I. The reactions are performed as follows.

1. Folin-Marenzi Reaction on Paper (*a*, *b*) (*20*)—The dried paper is sprayed with the Folin reagent diluted with 4 volumes of water. The paper, when still wet, is then passed over fumes of concentrated ammonia which has been poured in a large dish. The Folin-Marenzi reaction with bisulfite is performed similarly to the above procedure except that the Folin reagent is diluted with 4 volumes of 10 per cent sodium bisulfite. A positive reaction is shown by the appearance of a blue spot which persists for many days. The reaction is sensitive to 5 μg. of cysteine or cystine.

2. KI + HCl Reaction (*c*)—A fresh solution of 20 gm. of KI in 100 ml. of 2 N HCl is sprayed on the dried paper (1). The reaction appears slowly within an hour in the form of a red-brown spot on a yellow background. It is produced by the oxidation of HI to free iodine by some partially oxidized sulfur derivatives which in the same time are reduced to a lower level of oxidation. The limit of sensitivity is 5 μg. of cysteinesulfinic acid or hypotaurine.

3. Reaction with $FeCl_3$ (*d*) (*3*)—This reaction is specific for the sulfinic group, it is not very sensitive (30 μg. of hypotaurine are required), and does not appear if the paper contains traces of collidine-lutidine. A suitable amount of the suspected compound is eluted from the chromatogram, dried on another paper, and sprayed with a solution of 10 per cent $FeCl_3$. By this procedure a positive reaction is obtained even after the use of collidine-lutidine solvent and appears as a rusty spot on a yellow background.

4. $HgCl_2$ Reaction (*e*)—After evaporation of the solvent the paper (preferably strips of paper) is sprayed with a 2 per cent solution of $HgCl_2$ mixed with an equal volume of 0.1 M sodium acetate buffer of pH 5. After 2 minutes the paper is immersed in a tank of running tap water in such a way that it is completely immersed in a vertical position without touching the walls of the tank. After 15 minutes of thorough washing the paper is immersed in a vessel containing H_2S-saturated water. A black spot appears in a few seconds at the position occupied by a compound able to produce an insoluble complex with Hg.

5. Iodoplatinate Reaction (*f*)—This reaction is used following the procedure indicated by Toennies and Kolb (21).

When the above tests were applied to our chromatograms, compound 3 gave strongly positive reactions only with KI + HCl, $HgCl_2$, and iodoplatinate reagents; all of the other reactions were clearly negative. As shown by Table I this sequence of reactions is given by the following sulfur compounds: R—SO_2—S—R, R—SO—R, R—SO_2—SH. The first two of these structures could not be ascribed to compound 3 since cystamine disulfoxide and lanthionamine sulfoxide have different chromatographic coordinates. On this basis it was tentatively concluded that compound 3 was the thiosulfonate analogue of taurine; *i.e.* thiotaurine.

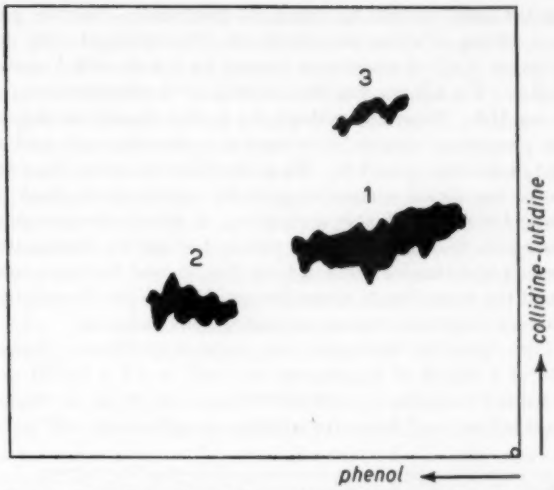

FIG. 1. Two-dimensional chromatogram of $\frac{1}{25}$ of the urine of 24 hours of two rats on a diet supplemented with 12 per cent cystine. Sample applied on the right lower corner. The phenol front was run from right to left; that for collidine-lutidine, upward. 1, taurine; 2, hypotaurine; 3, presumed thiotaurine.

TABLE I

Detection of sulfur derivatives on paper chromatograms

Compound*	Folin-Marenzi		KI + HCl	$FeCl_3$	$HgCl_2$	Iodoplatinate
	Without bisulfite	With bisulfite				
	a	*b*	*c*	*d*	*e*	*f*
R—SH	+	+	−	−	+	+
R—S—S—R	−	+	−	−	+	+
R—SO_2H	−	−	+	+	−	+
R—SO_3H	−	−	−	−	−	−
R—SO_2—S—R†	−	−	+	−	+	+
R—SO_2—SH	−	−	+	−	+	+
R—S—SO_3H	−	−	−	−	−	−
R—S—R	−	−	−	−	+‡	+
R—SO—R	−	−	+	−	+§	+
R—SO_2—R¶	−	−	+	−	−	+

* R represents CH_2—$CH(NH_2)$—COOH or CH_2—CH_2—NH_2.

† Reaction for these compounds when spotted on the paper. During chromatography they are changed to R—SO_3H + R—S—S—R.

‡ Other thioethers like methionine and carboxymethylcysteine give negative tests.

§ Other sulfoxides like methionine sulfoxide give negative test.

¶ Only lanthionine sulfone has been assayed.

Preparation of Thiotaurine—A sample of synthetic thiotaurine was needed to support the identification of compound 3 as thiotaurine. Thiotaurine had not previously been prepared; its occurrence in a biological medium had been suggested by Sörbo on the basis of studies on transulfuration of hypotaurine (6) in liver homogenates. Since Sörbo had been able to prepare alaninethiosulfonate by dismutation of cystine disulfoxide by H_2S (10), we attempted the preparation of thiotaurine by apply-

ing the same reaction to cystamine disulfoxide. For this purpose, 50 mg. of cystamine disulfoxide dihydrochloride (12) dissolved in 1 ml. of water were treated for 1 hour with a stream of H_2S. The solution was then aerated for 15 minutes to remove excess H_2S. Paper chromatography in two dimensions showed the presence of taurine, hypotaurine, cystamine, and another spot in the area of spot 3. When this spot was eluted from the paper, the eluted compound gave the reactions described by Sörbo for a thiosulfonate derivative. A mixed chromatogram made with the urine of rats fed with cystine and the dismutation product of cystamine disulfoxide by H_2S, showed that compound 3 and the thiosulfonate derivative produced in the dismutation reaction exhibited identical chromatographic behavior.

Pure crystalline thiotaurine was prepared as follows. A solution of 1 mmole of hypotaurine in 1 ml. of 0.2 N NaOH was added to 1.5 mmoles of sulfur and refluxed with 20 ml. of ethanol. After boiling for 2 hours the solution was placed at −20° overnight. The crystalline precipitate obtained was washed with carbon disulfide to remove unchanged sulfur. It was then recrystallized by dissolving it in the minimal amount of water and adding 30 ml. of absolute ethanol. After a day at −20°, 0.7 mmoles of pure crystalline thiotaurine were collected. M.p. was 213–214°.

$C_2H_7O_2NS_2$

Calculated: N 9.9, S 45.4

Found: N 9.6, S 45.6

Cyanolysis of the synthetic product gave products consistent with the expected properties of pure thiotaurine. Other details of the preparation and of the properties of the compound are described elsewhere (22). Synthetic thiotaurine was indistinguishable from compound 3 on paper chromatography. Fig. 2 describes a chromatogram of the urine of rats fed cystine added to 50 μg. of synthetic thiotaurine. In this chromatogram, which may be compared with that of Fig. 1, spot 3 is much larger and no separation into two components may be observed in either direction.

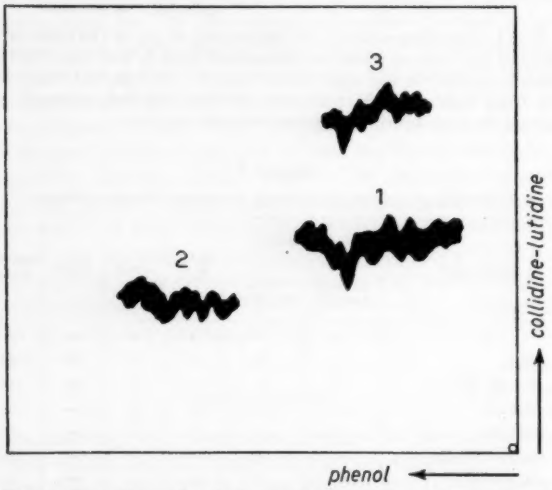

FIG. 2. Two-dimensional chromatogram of $\frac{1}{50}$ of the urine of 24 hours of two rats on a diet supplemented with 12 per cent cystine, added with a synthetic sample of pure thiotaurine. The same conditions as in Fig. 1; 50 μg. of thiotaurine have been added to the sample before chromatography.

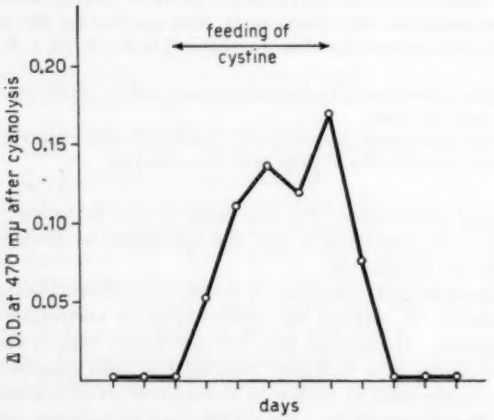

FIG. 3. Cyanolysis reaction for thiosulfonate (6) applied to 0.5 ml. of the 24-hour urine collection of two rats on a diet with and without added 12 per cent cystine.

Cyanolysis Reaction on Eluted Compound and on Urine—Gutman (23) has shown that thiosulfonate derivatives yield thiocyanate when treated with cyanide in alkaline medium. This reaction has been used as a qualitative and quantitative method for the estimation of thiosulfonates even in the presence of thiosulfate (6). When compound 3, eluted from the paper by a procedure similar to the one described above, was cyanolysed according to Sörbo (6), a strong positive reaction was obtained. Furthermore, paper chromatography of the reaction mixture showed that compound 3 was totally converted into hypotaurine by cyanolysis. The release of thiocyanate and hypotaurine by cyanolysis is consistent with the formulation of equation 1.

$$NH_2—CH_2—CH_2—SO_2—SH + CN^- \rightarrow NH_2—CH_2—CH_2—SO_3H + SCN^- \quad (1)$$

The reaction of cyanolysis was then applied to the urine in order to establish the occurrence of thiotaurine before the

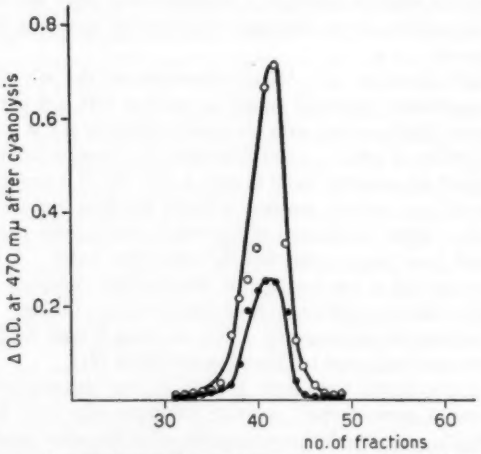

FIG. 4. Ion-exchange chromatogram by the method of Stein (23) with the use of citric acid as eluent (2) of the urine of rats on a diet containing 12 per cent of cystine. Fractions analyzed by cyanolysis according to Sörbo (6). The blank values have been subtracted. ●, 5 ml. of 24-hour urine of two rats; ○, the same sample of urine added with 600 μg. of synthetic thiotaurine.

chromatographic run. Although, as already stated, the procedure when applied to urine is not rigorously quantitative, we obtained consistent data regarding the excretion of a thiosulfonate derivative after ingestion of cystine. The result of one of these experiments is shown in Fig. 3. This finding has particular interest since it excludes the possibility of an artificial origin of thiotaurine during the course of the chromatographic manipulations. The values obtained indicate an excretion of approximately 1 to 2 mg. per day per rat, which is in good agreement with the amount calculated by the visual evaluation of the intensity and area of the chromatographic spot, and with the more precise data obtained by column chromatography.

Column Chromatography—When submitted to the Stein technique (24) for the analysis of amino acids as adapted by Cavallini *et al.* (2) to the determination of the oxidation products of cystine and cystamine, synthetic thiotaurine gives a typical peak which falls in the same area occupied by taurine. Nevertheless, it is possible to detect thiotaurine in the presence of a large amount of taurine when the cyanolysis reaction is used instead of the ninhydrin reaction. Fig. 4 gives the first part of a chromatogram, made by the Stein technique using citric acid-NaCl solution as eluent (2), of 5 ml. of urine of rats fed with cystine. By this very selective technique a compound with the properties of thiotaurine is detected in the urine of rats. The amount of thiotaurine in the sample analyzed indicates an excretion of 1.12 mg. of thiotaurine per rat per day.

Moreover column chromatography permits exact determination of the amount of thiocyanate released by cyanolysis of natural thiotaurine. This determination, deemed necessary to confirm in the natural product the presence of only 1 atom of sulfur removable by cyanide, was made as follows. A sample of natural thiotaurine was obtained by elution of a number of chromatograms. The content of thiotaurine in the pooled eluates was estimated by column chromatography using the ninhydrin reagent and a solution of synthetic thiotaurine as standard. The amount of thiocyanate released by cyanolysis was found by subjecting an aliquot of the eluates to the procedure of Sörbo (6). The following results were obtained: thiotaurine, 1.78 μmoles; SCN^- released, 1.70 μmoles. These values indicate that natural thiotaurine contains 1 atom of removable sulfur per mole.

SUMMARY

An unknown sulfur-containing compound has been detected in the chromatograms of urine of rats fed a diet supplemented with L-cystine. The compound is readily converted to taurine by hydrogen peroxide. A thiosulfonate structure was suggested by application of a number of reactions devised for determining the level of oxidation of organic sulfur groups. Synthetic thiotaurine, the thiosulfonate analogue of taurine, was indistinguishable from the unknown compound by paper and ion exchange chromatography. These and other properties support the conclusion that the unknown is thiotaurine (aminoethylthiosulfonic acid).

REFERENCES

1. Cavallini, D., and Mondovì, B., *Giorn. biochim.*, **1**, 171 (1951).
2. Cavallini, D., De Marco, C., and Mondovì, B., *Ricerca sci.*, **24**, 1021 (1954).
3. Cavallini, D., Mondovì, B., and De Marco, C., *J. Biol. Chem.*, **216**, 577 (1955).
4. Cavallini, D., Mondovì, B., and De Marco, C., *Giorn. biochim.*, **1**, 465 (1952).
5. Awapara, J., *Colloque sur la Biochimie du Soufre*, Editions du Centre National de la Recherche Scientifique, Paris, 1956, p. 99.
6. Sörbo, B., *Biochim. et Biophys. Acta*, **24**, 324 (1957).
7. Lavine, T. F., *J. Biol. Chem.*, **113**, 583 (1936).
8. Clarke, H. T., *Org. Syntheses*, **20**, 23 (1940).
9. Clarke, H. T., *J. Biol. Chem.*, **97**, 235 (1932).
10. Sörbo, B., *Biochim. et Biophys. Acta*, **22**, 570 (1956).
11. Toennies, G., and Lavine, T. F., *J. Biol. Chem.*, **113**, 571 (1936).
12. Cavallini, D., De Marco, C., Mondovì, B., and Merucci, P., *Giorn. biochim.*, **1**, 454 (1952).
13. Cavallini, D., De Marco, C., Mondovì, B., and Stirpe, F., *Rend. accad. nazl. Lincei*, **18**, 552 (1955).
14. Schöberl, A., *Ber. deut. chem. Ges.*, **80**, 379 (1947).
15. Schöberl, A., and Wagner, A., *Z. physiol. Chem.*, **304**, 97 (1956).
16. Zahn, H., and Osterloh, F., *Ann. Chem. Liebigs*, **595**, 237 (1955).
17. Gabriel, S., *Ber. deut. chem. Ges.*, **24**, 1110 (1891).
18. Cavallini, D., De Marco, C., and Mondovì, B., *J. Biol. Chem.*, **230**, 25 (1958).
19. Dent, C., *Biochem. J.*, **43**, 169 (1948).
20. Cavallini, D., Mondovì, B., and De Marco, C., *Biochim. et Biophys. Acta*, **18**, 122 (1955).
21. Toennies, G., and Kolb, J. J., *Anal. Chem.*, **23**, 823 (1951).
22. Cavallini, D., De Marco, C., and Mondovì, B., *Bull. soc. chim. biol.*, in press.
23. Gutman, A., *Z. anal. Chem.*, **47**, 294 (1908).
24. Stein, W. H., *J. Biol. Chem.*, **201**, 45 (1953).

The Metabolism of Serotonin (5-Hydroxytryptamine)*

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Serotonin has assumed an important role as the focal point about which much current neurochemical investigation is centered. The fact that its methylated derivatives and some compounds which antagonize its action can cause hallucinations has even led to the hypothesis that some defect in the body's ability to metabolize serotonin might be the underlying cause of schizophrenia. Yet relatively little is known about its metabolic fate in the body and 5-hydroxyindoleacetic acid, the product of oxidative deamination, is the only metabolite so far established (1). Although 5-hydroxyindoleacetic acid is allegedly the major metabolite, it represents only 33 per cent in the rat, 20 per cent in man, and 1.5 per cent in the rabbit, of administered serotonin (2). Experiments *in vitro* with rat liver and kidney preparations have also shown that less than 30 per cent of serotonin is converted to 5-hydroxyindoleacetic acid (1). Other pathways of metabolism have been suggested (3) and some unidentified indole derivatives in urines of patients with carcinoid syndrome and perfused rat livers have been reported (4–6). In addition, Chadwick and Wilkinson (7) have provisionally identified a metabolite of 5-hydroxytryptamine in rat liver homogenates as being the ethereal sulfate derivative and the oxytocic activity of normal human urine has been attributed to the presence of unchanged 5-hydroxytryptamine and its methylated derivatives (8).

EXPERIMENTAL

Materials and Methods

Compounds—5-Hydroxytryptamine-β-C^{14}-creatinine sulfate (see Fig. 5), with an activity of 184 μc. per gm., was prepared from material with a specific activity of 2 mc. per gm. by a 10-fold dilution with nonactive material and recrystallization to constant specific activity.

5-Hydroxytryptamine-*O*,*N*-dibenzoate: 5-Hydroxytryptamine creatinine sulfate, 0.5 gm., was benzoylated with 2.2 equivalents of benzoyl chloride and the resulting precipitate recrystallized from 75 per cent ethanol to yield 0.25 gm. of 5-hydroxytryptamine-*O*,*N*-dibenzoate as colorless needles, m.p. 172–3°. (Found C 74.92, H 5.25, N 7.29; $C_{24}H_{19}O_3N_2$ requires C 75.16, H 5.0, N 7.31 per cent.)

N-acetyl-5-hydroxytryptamine: Acetylation of 5-hydroxytryptamine by standard methods was found to give a mixture of *N*-acetyl- and *O*,*N*-diacetyl-5-hydroxytryptamine. The former could be distinguished chromatographically by virtue of its free hydroxyl group and was found to have an R_F of 0.75 in Solvent A (See Table I).

* The authors wish to thank Mrs. Gertrude H. Britton, whose generous financial support enabled this study to be undertaken.

Synthesis of the *N*-acetyl derivative was attempted by acetylation (glacial acetic: acetic anhydride, 1:1) of 5-hydroxytryptamine benzyl ether, 500 mg., (R_F 0.8 in Solvent A) to give 200 mg. of yellow crystals of 5-benzyloxy-3β-*N*-acetylaminoethylindole (R_F 0.54 in Solvent A). Debenzylation in ethanolic solution was accomplished with 10 per cent palladium-charcoal as a catalyst and hydrogen at room temperature for 2 hours. After removal of the catalyst the filtrate was evaporated at reduced pressure to leave a clear gum. Paper chromatography revealed the gum to be an indole with a free hydroxyl group, R_F 0.71 and 0.81, in Solvents A and B respectively, presumably *N*-acetyl-5-hydroxytryptamine. Attempts to crystallize the gum, however, were unsuccessful.[1]

5-Hydroxyindoleaceturic acid: An attempt was made to synthesize 5-hydroxyindoleaceturic acid from 5-hydroxyindoleacetic acid, 250 mg., and glycine ethyl ester hydrochloride, 180 mg., by the method of Sheehan and Hess (9). Attempts to crystallize the glycine conjugate from the reaction mixture failed but the presence of a phenolic indole derivative was demonstrated chromatographically with R_F 0.23 and 0.86 in Solvents A and B respectively.[1] This was shown to be 5-hydroxyindoleaceturic acid since it gave an orange spot when sprayed with *p*-dimethylaminobenzaldehyde in acetic anhydride demonstrating azlactone formation which is characteristic of aceturic acids (10). This reaction was not given by 5-hydroxyindoleacetic acid or any of the other metabolites.

Animals—Female Wistar rats of 200 to 250 gm. and female rabbits of Dutch or albino strains weighing 2.5 to 3 kg. were used. Serotonin and 5-hydroxyindoleacetic acid in aqueous solution were administered by intraperitoneal injection unless otherwise stated. Animals were fed on a standard diet with water *ad libitum* and kept in metabolism cages while under experiment.

Analytical Methods—Glucuronic acid and ethereal sulfate in urine were determined by the methods of Paul (11) and Sperber (12), respectively. Results are given in Table VI. 5-Hydroxyindoleacetic acid was measured colorimetrically (13) and serotonin fluorimetrically (14). For all fluorimetric determinations an Aminco-Bowman spectrophotofluorimeter was used.

Chromatographic Methods—For the detection of metabolites in urine and urine extracts descending chromatography with Whatman No. 1 or No. 4 paper was used. The solvents, R_F values and color reactions of reference compounds employed are given in Table I. Radioactive chromatograms were used to produce radioautographs.

For partial fractionation of metabolites, an aluminum oxide column 2 × 25 cm. was employed (8). Further fractionation

[1] Similar difficulties in obtaining crystalline derivatives of serotonin have been encountered by other workers (29).

TABLE I

R_F values and color reactions of some compounds related to 5-hydroxytryptamine

Solvent systems used were: *A*, propan-1-ol saturated with aqueous. NH_3 solution with Whatman No. 1 paper run for 14 hours; *B*, *n*-butanol-acetic acid-water (4:1:5) with Whatman No. 1 paper run for 14 hours; *C*, ethyl methyl ketone-2-*N*-NH_3 solution (2:1) with Whatman No. 4 paper run for 3 hours. For excitation of fluorescence an unfiltered ultraviolet lamp was used; Q means the background fluorescence of the paper was quenched. The sprays used for detecting compounds on paper were: Gibbs' reagent, consisting of 2 per cent ethanolic solution of 2,6 dichloroquinonechloroimide followed by saturated aqueous $NaHCO_3$; Naphthanil diazo blue B (tetrazotized di-*o*-anisidine) 3 per cent solution plus borate buffer pH 9. (3:2); Ehrlich's reagent, *p*-dimethylaminobenzaldehyde 0.5 per cent solution in 1.5 N HCl.

	R_F values in solvent			Fluorescence	Color of spots on paper with		
	A	B	C		Ehrlich's reagent	Gibbs' reagent	Naphthanil diazo blue
Indole	0.94	0.97	0.97		Pink	Red	Brown
Indole-3-acetic acid	0.35	0.95	0.12		Purple	None	None
Indole-3-proprionic acid	0.42	0.95	0.19		Purple	None	None
Indole-3-*n*-butyric acid	0.50	0.95	0.26		Blue	None	None
Indican	0.40	0.43	0.56		Brown		None
L-Tryptophan	0.26	0.62	0.08	Light blue	Pink	None	None
5-Hydroxytryptophan	0.11	0.42	0.03	Light blue	Blue	Blue	Blue
Dihydroxy phenylalanine	0.06	0.33	0.00	Blue	Pale green	Blue	None
Tryptamine	0.72	0.70	0.94		Purple		Yellow
5-Hydroxytryptamine	0.48	0.54	0.86	Light blue	Blue	Blue	Blue
N-methyl-5-hydroxytryptamine	0.73	0.49	0.83	Light blue	Blue	Blue	Blue
N-dimethyl-5-hydroxytryptamine	0.92	0.62	0.91	Light blue	Blue	Blue	Blue
5-Hydroxyindole-3-acetic acid	0.15	0.80	0.03	Light blue	Blue	Blue	Blue
N-acetyl-5-hydroxytryptamine	0.75	0.81	0.86		Blue	Blue	
Bufotenidine	0.20	0.59	0.15		Blue	Blue	Blue
Bufothionine	0.24	0.51	0.34		Purple		None
Dehydrobufotenine	0.46	0.76	0.09	Q	Purple		
5-Benzyloxy-3β-N-benzyl *N*-methylaminoethylindole	0.90	0.90	0.97		Blue	None	None
5-Benzyloxy-3β-dimethylaminoethylindole	0.89	0.76	0.97		Blue	None	None
5-Benzyloxyaminoethylindole sulfate	0.80	0.81	0.96		Blue	None	None
5-Benzyloxy-3β-*N*-acetylaminoethylindole	0.54	0.74	0.64		Blue		None
5-Hydroxyindoleaceturic acid	0.23	0.84			Blue	Blue	Blue

was accomplished with a BW-200 (Brown Company) cellulose column 2 × 25 cm. with the use of a one-phase solvent system of *n*-propanol saturated with ammonia.

For final fractionation separation on Whatman No. 1 paper with the solvents described in Table I was followed by ethanolic elution of strips. After removal of the ethanol at reduced pressure, aqueous solutions of the residues were employed for fluorimetric and pharmacological tests.

Measurement of Radioactivity—Measurements were carried out on solid samples of "infinite thickness" on nickel planchetes with an end window counter tube, the background of which was 20 to 25 c.p.m. The specific activities were determined by comparison with a stable polymer reference. A sample of 1 sq. cm. containing 0.1 μc. of C^{14} per gm. of substance gave approximately 270 c.p.m.

Urine and Tissues—Since it has been shown by Parke (15) that there is no significant difference in the results obtained by wet combustion of urinary residue to $BaCO_3$ (16) and those obtained by counting the solid residue obtained by evaporation of the urine directly on the planchetes under infrared lamps, the latter method was employed.

The radioactivity of the tissues was measured in a similar way by drying tissue homogenates directly on the planchetes. The results for the various tissues are shown in Table II; Fig. 1 shows the average rate of elimination of radioactivity in the urine.

Pharmacological Methods—Pharmacological characterization of serotonin and its derivatives was accomplished with the use of the oxytocic response of the isolated estrus rat uterus and the antagonism of this response by lysergic acid diethylamide and its bromo derivative. The tissue was suspended in an oxygenated organ bath containing Tyrode solution at 30°.

RESULTS

Rate of Excretion and Distribution in Tissues of Administered 5-Hydroxytryptamine-β-C^{14}-creatinine Sulfate—The rate of excretion of metabolites in the urine and feces after administration of 5-hydroxytryptamine-β-C^{14}-creatinine sulfate to rats and rabbits was measured by the activity present (Table II). More than 50 per cent of the activity appeared in the urine within 24 hours and the rate of elimination is shown graphically in Fig. 1.

Excretion of a small part of the dose in the feces was established not by the activity of the feces alone, which may have been subjected to urinary contamination, but also by the activity of the lower gut contents.

The percentage distribution of a dose of exogenous serotonin in the tissues of rats and rabbits is given in Table II.

Identification of Metabolites—In a preliminary experiment one rat was dosed with 10 mg. of C^{14}-5-hydroxytryptamine (200 μc.) and three rats with 10 mg. each of nonactive material. The 24-hour urines were collected, combined, and acetone extracted by the method of Bumpus and Page (8). Chromatography (Solvent A) of the concentrate revealed the presence of seven

TABLE II

Distribution in tissues and excretion of (C^{14}) 5-hydroxytryptamine

Animal No.	Rats			Rabbits	
	1	2	3	4	5
Weight (gm.)	270	260	180	2,500	2,500
Dose of (C^{14}) 5-hydroxytryptamine creatinine sulfate (mg.)	10	10	7	20	20
	% dose excreted in urine				
4 hr	2.9	6.25	3.27	69.1	71.09
24 hr	44.8	18.8	66.3	13.6	16.12
48 hr	8.1	*	1.37	4.65	4.15
72 hr			0.58	4.6	4.20
96 hr			0.57		3.35
Total	55.8	25.05	72.09	91.95	98.91
Feces	3.5	0.41	5.33	0.46	0.15
Stomach contents	0.01		0.0	0.46	
Small gut			0.10		
Gut contents	2.4		0.13	0.0	
Blood-red cells	5.0	3.25	0.53	0.61	
Blood-plasma			1.10	2.64	
Heart	0.07	0.32	0.05	0.0	
Lung	0.08	0.49	0.02	0.11	
Liver	0.50	2.80	0.38	0.0	
Kidney	0.20	0.44	0.05	0.08	
Spleen	0.15	0.19	0.03	0.27	
Brain	0.01	0.03	0.07	0.34	
Total	67.72	32.98	79.88	97.23	

* This animal died 48 hours after dosing.

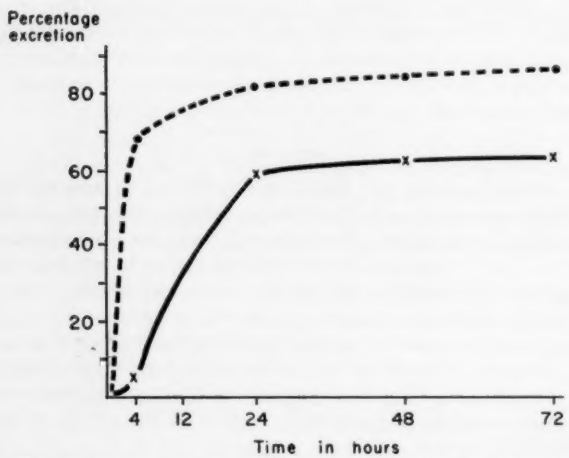

FIG. 1. Average urinary excretion of radioactivity following the administration of C^{14} serotonin to rats, X——X, and rabbits, ●---●.

indole spots and radioautography showed three of these to be radioactive (Fig. 2). These were 5-hydroxyindoleacetic acid, R_F 0.15; 5-hydroxytryptamine, R_F 0.48, and *N*-acetyl-5-hydroxytryptamine, R_F 0.75. The 5-hydroxyindoleacetic acid spot appeared to be separating into two components and an eluate of this area was resolved in Solvent B into two spots, 5-hydroxyindoleacetic acid, R_F 0.8, and 5-hydroxyindoleaceturic acid (positive azlactone spot R_F 0.84).

Further evidence for the formation of 5-hydroxyindoleaceturic acid was obtained by dosing three rats, each with 40 mg. of 5-hydroxyindoleacetic acid. The 24-hour urines were collected and found to give a blue color with 2,6-dichloroquinonechloroimide but did not give a positive naphthoresorcinol reaction. Chromatography of these urines disclosed two indole metabolites which proved to be 5-hydroxyindoleacetic acid, R_F 0.8, and 5-hydroxyindoleaceturic acid, R_F 0.85 (Solvent B), the latter giving a positive azlactone reaction (10).

The presence of two other metabolites was observed in the urine of Rat 1 (Table II). The 24-hour urine was ether extracted at pH 5 and 8. The extracts and aqueous residue were chromatographed and radioautographs obtained (Fig. 2). These showed the presence of 5-hydroxyindoleacetic acid and *N*-acetyl-5-hydroxytryptamine in the acid extract, little or nothing in the basic extract, and four other metabolites in the aqueous residue. Removal of the 5-hydroxyindoleacetic acid by extraction left the 5-hydroxyindoleaceturic acid, R_F 0.23, in the aqueous layer. In addition there was some 5-hydroxytryptamine, R_F 0.48, and

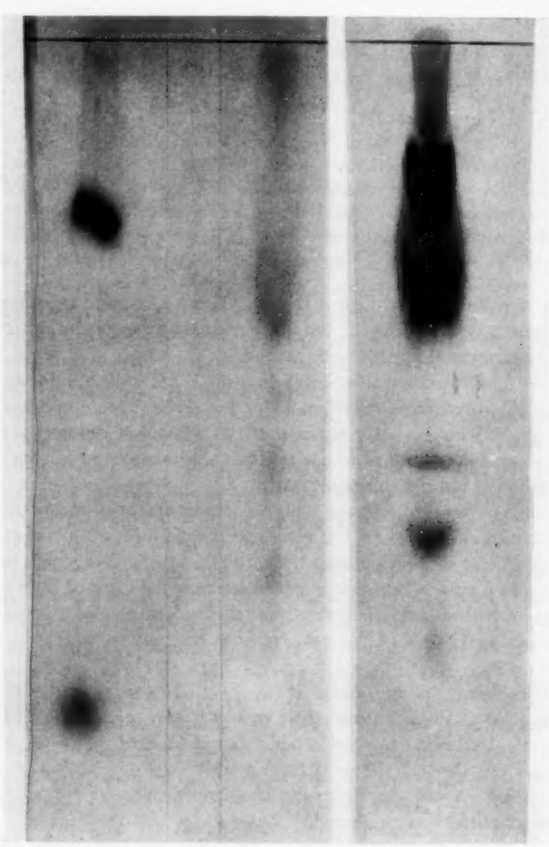

FIG. 2. Radioautographs of indole extracts from, *left*, rat urine, and *right*, rabbit urine. The extract from rat urine has been divided into an ether fraction, extreme left, containing 5-hydroxyindoleacetic acid and *N*-acetyl-5-hydroxytryptamine; and an aqueous fraction containing the other metabolites.

two small spots at R_F 0.35 and 0.60 which gave positive color reactions for indoles. The latter of these also gave a blue color when sprayed with 2,6-dichloroquinonechloroimide or tetrazotized *o*-dianisidine (Table I) and a red color with diazotized sulfanilic acid, showing it to be a hydroxy indole with an intact amino group in the side chain. Yet its chromatographic properties and fluorescent spectra (λ activation 305 mμ and λ emission 360 mμ with maximal sensitivity at pH 7) showed that it was not unchanged serotonin (Table III). The most probable structure to fit these observations would be that of an oxidative product of serotonin, either an oxindole or dihydroxy derivative. The results of color tests for dihydroxy indoles, however, proved inconclusive.

Quantitative estimations had shown some increase in the excretion of glucuronic acid after administration of serotonin and the possibility that the former of these metabolites might be a glucuronide was confirmed in the following manner. Five rats were dosed with 20 mg. each of C^{14}-5-hydroxytryptamine, the 24-hour urines collected and subjected to the systematic lead acetate precipitation method (17), and a small amount of glucuronide gum obtained from the basic lead acetate precipitate. A naphthoresorcinol reaction on the gum was masked by an intense red color which developed in the aqueous phase. The residual gum was chromatographed and showed the presence of two indoles, R_F 0.35 and 0.5 (Solvent A). The latter proved to be serotonin but the former gave a negative Gibbs' reaction (See Table I) showing that the hydroxyl group was not free. An eluate of this indole was found to be radioactive and to give a positive naphthoresorcinol reaction, indicating that it was the ether glucuronide of 5-hydroxytryptamine.

The metabolic fate of serotonin in the rabbit was elucidated in a similar fashion with the urines obtained from Animals 4 and 5 (Table II). Autoradiography and chromatography disclosed the major metabolite to be 5-hydroxyindoleaceturic acid (positive azlactone spot R_F 0.23 and 0.84 in solvents A and B, respectively), with some free 5-hydroxyindoleacetic acid. Small amounts of serotonin and its *N*-acetyl derivative were also present (Fig. 2).

Quantitative Results—Since many of the possible metabolites were difficult to obtain in sufficient quantity for standard isotopic dilution techniques, quantitative estimation of metabolites had to be made by scanning radioactive chromatograms with a sensitive end-window counter and plotting the activity against R_F values. The percentage excretion of different metabolites could then be calculated (Table IV) since the total activity of the urine had previously been estimated (Table II). This method was satisfactory for the acetone extracts which were found to contain the total activity of the urine. However, in order to estimate the percentages of 5-hydroxyindoleacetic and -aceturic acids, which by this method were counted together, their R_F values in Solvent A being very similar, use was made of the greater ether solubility of the unconjugated acid. In Experiments 1 and 4, therefore, known quantities of ether extracts and aqueous residues containing known proportions of the total activity were chromatographed and counted separately.

Quantitative results of glucuronide and ethereal sulfate formation were estimated with the use of rats and rabbits (Table V). 5-Hydroxyindoleacetic acid caused no increased excretion but 5-hydroxytryptamine gave rise to a slight increase in both species due to the formation of an ether glucuronide.

Metabolic Fate of Endogenous Serotonin in Patients with Carcinoid Syndrome—Three gallons of urine from two patients with

TABLE III

Fluorescent spectra

	Activation maximum	Fluorescent maximum	pH	Ultimate sensitivity
				μg./ml.
Indole	280	355	7	0.002
Indican	300	400	7	0.001
5-Hydroxytryptophan	310	350	7	0.005
5-Hydroxytryptamine	295	540	2	0.003
5-Hydroxyindole-3-acetic acid	300	355	7	0.002
N-methyl-5-hydroxytryptamine	310	355	7	0.004
N-dimethyl-5-hydroxytryptamine	310	355	7	0.002
N-acetyl-5-hydroxytryptamine	310	370	7	0.001

TABLE IV

Elimination of metabolites of serotonin in urine of animals receiving 5-hydroxytryptamine-β-C^{14}

	R_F (solvent A)	Experiment No.			
		0	1	3	4
5-Hydroxyindoleacetic acid	0.15	43.5	25	34.8	5
5-Hydroxyindoleaceturic acid	0.23		14.6		78
5-Hydroxytryptamine glucuronide	0.35		trace	trace	0
5-Hydroxytryptamine	0.48	5.5	7.3	9.4	2
? Oxidation product	0.60		3.5	2.5	5
N-acetyl-5-hydroxytryptamine	0.75	11	5	25.4	2
Sum of metabolites = total activity of urine		60	55.4	72.1	92

TABLE V

Quantitative aspects of conjugation of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Compound	Animals	Dose*	Conjugation (% of dose) with†		
			Glucuronic acid	Ethereal sulfate	Glycine‡
		mg./kg.			
5-Hydroxytryptamine	Rats	80	10 (0 to 30)	2.4 (0 to 4.4)	+
5-Hydroxytryptamine	Rabbits	50	4 (0 to 12)		++
5-Hydroxyindoleacetic acid	Rats	250	0 (0 to 0)	0 (−3.6 to +6.6)	+

* This represented the maximal dose which could be well tolerated.

† Results are quoted as the average for three animals, with range in parenthesis.

‡ Demonstrated chromatographically by colored azlactone formation (10).

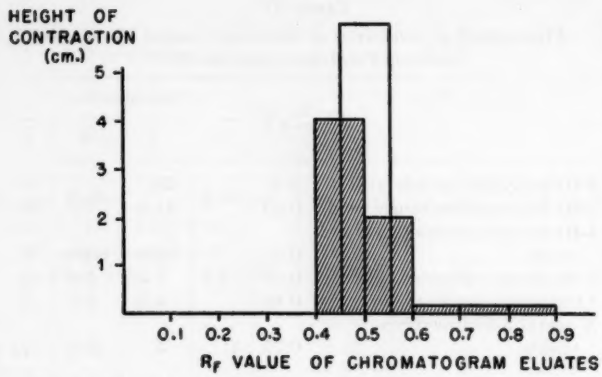

FIG. 3. Oxytocic activity of urinary metabolites of serotonin after separation by paper chromatography (Solvent A). When eluates according to R_F value were tested, maximal activity was obtained in the two fractions R_F 0.4 to 0.5 and 0.5 to 0.6 (hatched areas). When indole spots were eluted this activity (unhatched area) was found to be associated with one spot, R_F 0.48 (0.44 to 0.53), which proved to be serotonin.

carcinoid syndrome containing 480 mg. of 5-hydroxyindoleacetic acid were acidified to pH 5 with glacial acetic acid and concentrated at reduced pressure at 40° to 3 l. Urease was added and the concentrate incubated at 37° for 12 hours and then reduced to 500 ml. On the addition of 5 l. of acetone a precipitate formed and was filtered off. The filtrate was then finally reduced to a volume of 20 ml. Chromatography of this concentrate revealed the presence of at least six indole derivatives and urea, R_F 0.32 (Solvent A).

An aluminum oxide column was used to fractionate 3 ml. of this concentrate. The adsorbates were eluted with methanol diluted with increasing amounts of water as follows: 100 ml. of absolute methanol, 100 ml. of 75 per cent methanol, 100 ml. of 50 per cent methanol, 100 ml. of 25 per cent methanol; 50 ml. fractions were collected. Paper chromatography of the eight eluates showed a partial fractionation with most of the metabolites in the first three fractions, urea, and some hydroxyindoleacetic acid in the remaining fractions. Use was made of this to purify further the rest of the crude urinary concentrate.

The purified mixture of metabolites was then fractionated on a cellulose column with the use of a one-phase *n*-propanol-ammonia solvent. Twenty fractions of 10 ml. were collected, evaporated to dryness under reduced pressure, and isolation of the metabolite attempted. Fractions 2 and 3 contained *N*-acetyl-5-hydroxytryptamine. Fractions 4 and 5 contained 5-hydroxytryptamine and indican. Fractions 6, 7, 8, and 9 contained 5-hydroxyindoleacetic acid. These were identified by paper chromatography in two solvents; when the fractions containing 5-hydroxyindoleacetic acid were chromatographed in Solvent B, in addition to the spot for 5-hydroxyindoleacetic acid, R_F 0.80, there was another spot at R_F 0.86, 5-hydroxyindoleaceturic acid.

Isolation of Metabolites from Urine of Patients with Carcinoid Syndrome—Serotonin was isolated from Fractions 4 and 5 which were pooled and benzoylated with excess benzoyl chloride, the precipitate filtered off and recrystallized from ethanol to give about 2 mg. of 5-hydroxytryptamine-*O*,*N*-dibenzoate, m.p. and mixed m.p. with authentic material, 171 to 173°.

Attempts to isolate *N*-acetyl-5-hydroxytryptamine and 5-hydroxyindoleacetic acid from the relevant fractions by forming benzoates or *p*-toluene sulfonates were unsuccessful.

Characterization of Metabolites—Some of the urinary concentrate, 0.5 ml., was streaked on Whatman No. 1 paper and separation of metabolites achieved with Solvent A. A control strip from the side of the chromatogram was sprayed and sections of the paper corresponding to the metabolites were eluted with ethanol. The eluates were evaporated to dryness under reduced pressure and the residues dissolved in 1 ml. of water. These aqueous solutions were used for determination of the fluorescent spectra of the metabolites (Table IV), oxytocic activity (Fig. 3) and chromatographic identification in two solvents (Table I). In this way it was possible to identify the following five indole derivatives in order of their increasing R_F value in Solvent A: 5-hydroxyindoleacetic acid, R_F 0.17; 5-hydroxyindoleaceturic acid, R_F 0.23; indoxyl potassium sulfate, R_F 0.40; 5-hydroxytryptamine, R_F 0.48; and *N*-acetyl-5-hydroxytryptamine, R_F 0.72. A small amount of another unidentified indole derivative was present (R_F 0.81 and 0.86 in Solvents A and B, respectively).

DISCUSSION

Exogenous serotonin is rapidly metabolized by rats and rabbits and excreted, 50 to 80 per cent being eliminated in the urine within 24 hours (Fig. 1). The only exception in Experiment 2, where excretion was 25 per cent in 24 hours, the animal developed paresis of the hind legs, became comatose, and died. Enlarged pale kidneys were revealed at necropsy.

Excretion of 0.5 to 5 per cent of a dose of serotonin via the gut was confirmed by the activity of the lower gut contents (Table II). Since these animals were dosed by intraperitoneal injection this must have been due to excretion. This excretion was accompanied by increased gut motility.

Serotonin has been reported to cause hypernea in some animal species (18, 19) and a moderate tachypnea was observed during the course of these experiments which was of interest since the specific activity of the lung tissue was found to be fairly high, even 3 days after administration.

Platelets *in vivo* are not saturated with serotonin (20) and it has been shown (21) that they will take it up *in vitro*. This appears to be true *in vivo* since the activity of the blood following administration of C^{14}-serotonin was largely associated with the platelet containing fraction of the plasma and the level was maintained for some time, being 3.25 per cent after 2 days and 3.3 ± 1.7 per cent after 3 days (Table II). The small amount of activity associated with the red cells might be due to their ability to absorb serotonin (22) though to a smaller extent than platelets. This evidence suggests that serotonin taken up by the platelets may be protected from metabolism and elimination for several days.

Much evidence has been presented for the inability of serotonin to cross the blood-brain barrier. The present radioactive studies, however, suggest that a small amount of it may do so since the brains of two of the rats were found to contain significant amounts of activity and in one of the rabbits, 4 days after dosing, the brain had a higher specific activity than the other organs (Table II). The fact that this activity might be due to the entry of metabolites rather than serotonin, however, cannot be discounted.

Species Difference in Metabolism—Several reports have been made of species differences in serotonin metabolism (2, 23). Sanyal and West (24) found such in anaphylactic shock and

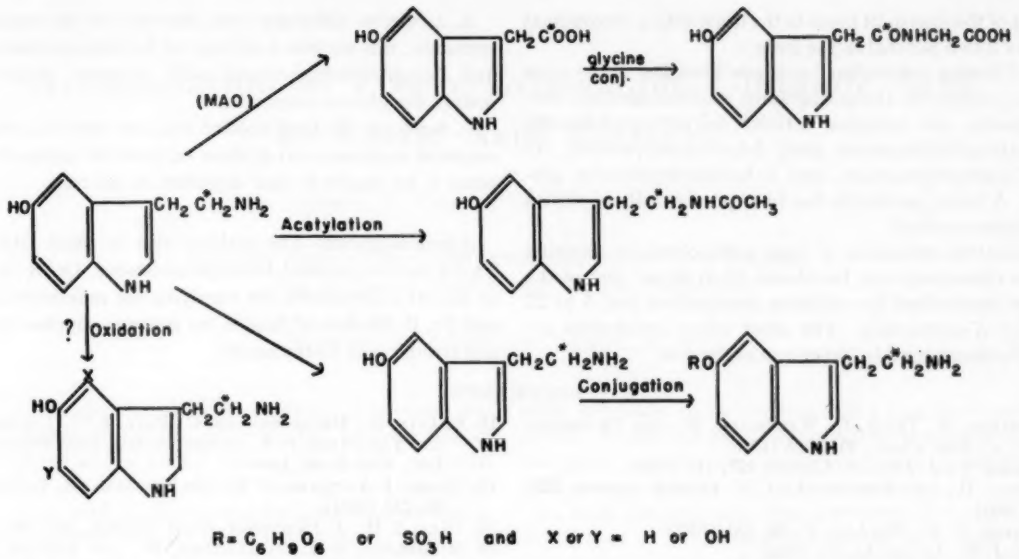

FIG. 4. Metabolism of 5-hydroxytryptamine-β-C^{14}. Major route arises from monoamine oxidase (MAO) activity but acetylation conjugation, and excretion unchanged are important alternatives. Oxidation is also a possibility.

Erspamer (2) noted that whereas carnivorous animals excreted 5-hydroxyindoleacetic acid as the major metabolite that herbivorous species excreted practically none. In the present work this species difference in the metabolism of serotonin has been confirmed. In both species oxidative deamination was the major pathway, but whereas all the rats excreted a mixture of 5-hydroxyindoleacetic acid and 5-hydroxyindoleaceturic acid, the rabbits excreted mainly the glycine conjugate (Table IV). This difference might account for the slightly more rapid excretion by rabbits (Fig. 1). Although there was no difference in glucuronide formation (Table V) there were some minor quantitative differences in the excretion of the other metabolites.

Metabolic Fate of Serotonin—As can be seen from Table V the major metabolites of serotonin in the urine of rats and rabbits are those that result from amine oxidase action. Over 50 per cent of the dose excreted in the urine is accounted for by 5-hydroxyindoleacetic and 5-hydroxyindoleaceturic acids. Subsequent glycine conjugation occurs to a much greater extent in rabbits than in rats.

Acetylation plays an important part in the metabolism of serotonin, 5 to 25 per cent of the urinary metabolite being in the form of *N*-acetyl-5-hydroxytryptamine. This product has no oxytocic activity (see Fig. 3).

Excretion of unchanged serotonin has been found to account for 5 to 9 per cent of the dose, and serotonin is certainly the most potent oxytocic compound present in urine (Fig. 3).

Conjugation with glucuronic acid and ethereal sulfate has been found to occur, though only to a small extent; the ether glucuronide of 5-hydroxytryptamine being formed. In comparison 5-hydroxyindoleacetic acid does not appear to form any ether conjugates, being excreted almost entirely as a mixture of the unchanged acid and its glycine conjugate.

One of the metabolites of norepinephrine has been shown to be 3-methoxy-4-hydroxy mandelic acid (25). This involves methylation of the phenolic hydroxyl group. No evidence of *O*-methylation of exogenous serotonin could be found in these experiments.

N-methylation of amino compounds *in vivo* has been shown *e.g.* *N*-methylation of histamine with subsequent excretion as *N*-methyl and *N*-dimethyl histamine in the urine (26). The possibility of serotonin being *N*-methylated is interesting since it has been shown that the *N*-methyl derivatives have hallucinogenic properties (27). However, in this study of the normal metabolic fate of exogenous serotonin no evidence has been found for the formation of methyl derivatives.

The possibility of further oxidation is of interest since it has been suggested by Dalgliesh (28) that serotonin might function through some more active derivative such as 5,6-dihydroxytryptamine. Although an exact characterization has not been possible, some evidence has been found in the present work that one of the minor metabolites of serotonin might be an oxidation product. Although this metabolite possessed little oxytocic activity, and other work in this laboratory has shown that 5,6-dihydroxytryptamine as a vasopressor is less potent than serotonin, it does not preclude the possibility that this derivative might be centrally active.

The metabolism of serotonin has proved to be complex and it may be possible to interpret the actions of some drugs, *e.g.* monoamine oxidase inhibitors, by the way they interfere with the normal metabolic pattern. Since it is possible that some psychoses may be due to abnormal metabolism of serotonin, elucidation of its normal metabolism has been a necessary preliminary to the study of these other problems.

SUMMARY

1. A study has been made of the metabolism of exogenous C^{14}-5-hydroxytryptamine in rats and rabbits.

2. The activity of various tissues following administration of C^{14}-5-hydroxytryptamine has been estimated. Major activity was found in the platelet-containing fraction of the plasma and significant activity was found in lung and brain tissue.

3. The excretion of activity after administration of the radioactive compound to rats and rabbits has been found to be 50 to

98 per cent of the dose in 24 hours in the urine with a concomitant excretion of 3 to 5 per cent in the feces.

4. The following metabolites have been identified in the urine of rats and rabbits by chromatography, radioautography, fluorescent spectra, and biological activity: 5-hydroxyindoleacetic acid; 5-hydroxyindoleaceturic acid; 5-hydroxytryptamine; *N*-acetyl-5-hydroxytryptamine, and 5-hydroxytryptamine glucuronide. A minor metabolite has been provisionally identified as an oxidation product.

5. Quantitative estimation of these metabolites, by scanning radioactive chromatograms, has shown 35 to 83 per cent of the dose to be metabolized by oxidative deamination and 5 to 25 per cent by *N*-acetylation. The other minor metabolites account for the remaining 5 to 10 per cent of the dose.

6. A species difference was observed in the metabolism of serotonin: rats excrete a mixture of 5-hydroxyindoleacetic acid and 5-hydroxyindoleaceturic acid, however, rabbits excrete mainly the glycine conjugate.

7. Serotonin has been isolated from the urine of patients with carcinoid syndrome and in these subjects the metabolic fate appears to be similar to that described in the rat.

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REFERENCES

1. Udenfriend, S., Titus, E., Weissbach, H., and Peterson, R. E., *J. Biol. Chem.*, **219**, 335 (1956).
2. Erspamer, V., *J. Physiol. London*, **127**, 118 (1955).
3. Blaschko, H., and Philpot, F. J., *J. Physiol. London*, **122**, 403 (1953).
4. Dalgliesh, C. E., *Biochem. J.*, **64**, 481 (1955).
5. Jepson, J. B., *Lancet*, **1**, 119, (1955).
6. Dalgliesh, C. E., and Dutton, R. W., *Biochem. J.*, **65**, 21p (1957).
7. Chadwick, B. T., and Wilkinson, J. H., *Biochem. J.*, **68**, 1p (1958).
8. Bumpus, F. M., and Page, I. H., *J. Biol. Chem.*, **212**, 111 (1955).
9. Sheehan, J., and Hess, G., *J. Am. Chem. Soc.*, **77**, 1067 (1955).
10. Gaffney, G. W., Schreier, K., DiFerrante, N., and Altman, K. I., *J. Biol. Chem.*, **206**, 695 (1954).
11. Paul, J., Ph.D. Thesis: University of Glasgow, 1951.
12. Sperber, I., *J. Biol. Chem.*, **172**, 441 (1948).
13. Udenfriend, S., Titus, E., and Weissbach, H., *J. Biol. Chem.*, **216**, 499 (1955).
14. Bogdanski, D. F., Pletscher, A., Brodie, B. B., and Udenfriend, S. *J. Pharmacol. Exptl. Therap.*, **117**, 82 (1956).
15. Parke, D. V., *Biochem. J.*, **62**, 339 (1956).
16. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. H., and Yankwich, P. F., *Isotopic carbon*, John Wiley and Sons, Inc., New York, 1949.
17. Kamil, I. A., Smith, J. N., and Williams, R. T., *Biochem. J.*, **50**, 235 (1951).
18. Page, I. H., *J. Pharmacol. Exptl. Therap.*, **105**, 58 (1952).
19. Michelson, A. L., Hollander, W., and Lowell, F. C., *J. Lab. Clin. Med.*, **51**, 57 (1958).
20. Hardisty, R. M., and Stacey, R. S., *J. Physiol. London*, **130**, 711 (1955).
21. Brodie, B. B., *Proceedings of the symposium on 5-hydroxytryptamine*, Pergamon Press, Ltd., New York, 1957, p. 71.
22. Stacey, R. S., *J. Physiol. London*, **132**, 39p (1956).
23. Nakai, K., *Nature*, **181**, 1734 (1958).
24. Sanyal, R. K., and West, G. B., *Nature*, **180**, 1417 (1957).
25. Shaw, K. N. F., McMillan, A., and Armstrong, M. D., *Federation Proc.*, **15**, 353 (1956).
26. Kapeller-Adler, R., and Iggo, B., *Biochim. et Biophys. Acta.*, **25**, 394 (1957).
27. Fabing, H. B., and Hawkins, J. R., *Science*, **123**, 886 (1956).
28. Dalgliesh, C. E., *Proceedings of the symposium on 5-hydroxytryptamine*, Pergamon Press, Ltd., New York, 1957, p. 60.
29. Speeter, M. E., Heinzelmann, R. F., and Weisblat, D. I., *J. Am. Chem. Soc.*, **73**, 5516 (1951).

Accumulation of Freely Extractable Glutamic Acid by Lactic Acid Bacteria*

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Gale (2, 3) initially described the existence in bacteria of a metabolism-dependent process achieving the accumulation of freely extractable amino acids. During the studies described here a number of additional reports have appeared citing examples of amino acid (4, 5), carbohydrate (6), and phosphate (7) accumulations in bacteria. The mechanism of these accumulations is unknown.

Christensen *et al.* (8) have observed that pyridoxal stimulates amino acid uptake by Ehrlich ascites tumor cells. This and a companion paper describe an investigation intended to determine whether vitamin B_6 deprivation influences the activity of a bacterial amino acid accumulation system. The lactic acid bacteria were selected for study in view of the ease with which the intracellular vitamin B_6 content can be varied in this group of organisms (9). A description of glutamic acid accumulation in several species is presented here. The following paper (10) summarizes experiments designed to test the effect of vitamin B_6 deficiency on the accumulation process.

EXPERIMENTAL

Cultures—Lactobacillus arabinosus 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60 were carried in stabs of glucose-yeast extract-agar (0.25 per cent, 1 per cent, 1.5 per cent), transferred monthly and stored at 4° between transfers. Cells used in accumulation experiments were grown in previously described completely synthetic media (11). For *L. arabinosus* the medium was modified to contain 500 mg. per l. of L-alanine and 100 mg. per l. of D-alanine. Vitamin B_6 was supplied as pyridoxamine (1 μg. per ml.) which was added as a sterile solution to the previously sterilized vitamin B_6-deficient medium. Sterilization was achieved by autoclaving at 15 pounds for 6 to 10 minutes, depending on the volume of medium, which varied between 1 and 8 l.

Inocula were grown at 30° for 16 to 20 hours in the appropriate synthetic medium. Since these experiments were performed during a comparative study designed to determine the effect of vitamin B_6 deficiency on accumulation ability (10), inoculum cultures were routinely grown without vitamin B_6 supplementation. To ensure vitamin depletion in these cells, the inoculum culture was separated from the stab culture by at least one previous transfer in the vitamin B_6-deficient medium. After incubation, the cells were centrifuged, resuspended in cold distilled water and added to the medium with 1.0 μg. dry weight of cells per ml. In the final mass culture, cells used in these experiments were grown at 30° under an atmosphere of carbon dioxide, in medium supplemented with pyridoxamine.

In most instances cells were harvested 12 to 13 hours after inoculation, at which time the cultures were near the end of the exponential phase of growth. They were chilled in ice and collected batch-wise in a refrigerated centrifuge. The cells were washed once in distilled water and then resuspended in cold water to a density of 20 mg. (dry) per ml. The density of the suspension was estimated from its absorbance at 640 mμ with the use of a curve relating dry weight and absorbance. In most experiments this value was checked by measuring the dry weight of an aliquot of the suspension. All descriptions of the weight of cells refer to the dry weight.

Uptake Conditions—Initially, experiments were performed with the buffered salt solution described by Gale (2). However, with the use of *L. arabinosus* at a cell density of 1.6 mg. per ml., this solution was found to be inadequately buffered, and, consequently, the amount of phosphate was increased to give a final concentration of 0.12 M. Thereafter experiments were carried out in 0.12 M phosphate, pH 6.5 containing glucose (0.028 M), glutamic acid (0.003 M), NaCl (0.014 M), and $MgSO_4$ (0.0023 M); 20 mg. of cells were used in a final volume of 12.5 ml. This will be referred to as the standard uptake conditions. The general procedure was to dispense buffer, L-glutamic acid, in most experiments uniformly labeled with C^{14}, and glucose to plastic centrifuge tubes. These were preincubated in a water bath at 37° to ensure temperature equilibration and then the thick cell suspension (20 mg. per ml.) was added to give a final cell concentration of 1.6 mg. per ml. All experiments included controls lacking either or both glucose and glutamate. In experiments involving variation in the time of incubation, the required number of identically constituted tubes were inoculated and after the requisite period of incubation each of these in turn was transferred to a prechilled rotor and centrifuged in the cold. Cells which had been added to uptake medium at 2° and centrifuged immediately, served as unincubated controls. The tubes were centrifuged at an average of 18,000 × *g* for 10 minutes. The supernatant buffer was poured off and the inside of the tube was carefully swabbed dry without righting the tube. Both the supernatant solution and the cell pellet were frozen immediately and stored in this condition until analyzed.

Preparation of Cell Extracts for Assay—A number of cell extraction procedures have been used, all of them yielding essentially identical extracts as judged by the analyses used in

* A preliminary report of some of this work has appeared (1). This study was supported by a grant (E-1487) from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

this study. In most experiments the cell pellet was thawed, macerated in the cold, and immediately suspended in 6 ml. of 80 per cent ethanol at 75°. The suspension was transferred to a glass centrifuge tube and heated at 75° for 10 minutes. The tubes were centrifuged and the supernatant removed to a beaker. The residue was resuspended in 6 ml. of ethanol-water and re-extracted for an additional 10 minutes. The combined supernatants were reduced to dryness in a current of air under an infrared lamp. The residues were taken up in water (2 ml. per 20 mg. of cells extracted) and centrifuged to remove insoluble material. In experiments in which the intracellular free amino acids were to be examined by paper chromatography, an aliquot of the solution equivalent to 20 mg. of cells was taken to dryness and resuspended in 0.3 ml. of saturated picric acid. The resultant suspension was clarified by centrifugation before application to paper. Extraction with water was carried out similarly except the temperature of extraction was that obtained in a boiling water bath. When examination of the acid-soluble nucleotide fraction was desired, extraction was achieved with 0.5 N perchloric acid. Ice-cold perchloric acid, 4 ml., was added to the thawed cell pellet. The suspension was transferred to chilled glass centrifuge tubes, allowed to stand for 25 minutes in an ice bath, and centrifuged in the cold for 10 minutes. The residue was re-extracted for 10 minutes in the cold with 4 ml. of 0.5 N perchloric acid. The extracts were freed of most of the perchlorate by adjusting to pH 6.5 with concentrated KOH in an ice bath. The precipitated potassium perchlorate was removed by centrifugation.

Analyses—L-Glutamic acid was measured manometrically by decarboxylation with an acetone powder of *Escherichia coli* E-26 prepared as described by Ayenger *et al.* (12). The samples were assayed with the use of 2 mg. of cell powder and 1 ml. of 0.15 M acetate buffer at pH 4.2 containing cetyltrimethylammonium bromide (3.7 mg. per ml.), in a final volume of 2.5 ml. L-Glutamine was slowly decarboxylated by the preparation used in all of these experiments. Paper chromatography showed, however, that only small amounts of glutamine occur in experimental cell extracts (at most 5 per cent of the glutamic acid present). Therefore, the error introduced by this lack of specificity is small. In subsequent studies, additional preparations of this powder have been devoid of activity toward glutamine. Recovery of known amounts of L-glutamic acid (measured in the presence and absence of the D-isomer in cell extracts) was 90 to 105 per cent and in most instances 95 to 98 per cent. The supernatant buffer containing 0.15 M phosphate was adjusted to pH 4.5 before assay for L-glutamic acid, but when it contained only 0.024 M phosphate it was assayed directly.

Radioactivity of cell extracts and supernatant buffer was determined with the use of a gas-flow counter (Nuclear-Chicago Corporation). Aliquots of samples were applied to circles of filter paper glued to aluminum planchets. All samples were plated in triplicate. The observed counts were adjusted to the count at infinite thinness by correcting for self-absorption due to paper, cell extract, and supernatant buffer as appropriate for the sample.

Changes in the freely-extractable amino acid content were determined by two-dimensional paper chromatography of cell extracts. The equivalent of 20 mg. of cells as a picric acid filtrate was applied to one corner of an 18 × 22 inch sheet of Whatman No. 1 paper. The paper was developed by the descending technique in chambers with the use of phenol-ammonia-water followed by lutidine-water. After drying at room temperature, the amino acids were visualized by spraying with a ninhydrin solution. A complete description of the pool and its variations will be presented elsewhere. To determine the extent of metabolism of accumulated, uniformly labeled glutamic acid, two-dimensional paper chromatograms of cell extracts and supernatant buffers were exposed to Kodak Industrial Type K x-ray film for 3 weeks.

Chemicals—The components of the growth medium were all commercially available substances. Uniformly labeled L-C^{14}-glutamic acid was obtained from the Nuclear-Chicago Corporation.

RESULTS AND DISCUSSION

Most of the general characteristics of the glutamic acid accumulation process were investigated in *L. arabinosus* with cells grown with an excess of pyridoxamine. As shown in Fig. 1*A*, L-glutamate accumulation depends on the presence of glucose and an elevated temperature of incubation. During incubation with glutamic acid alone, there is only a slight transient increase in the endogenous content of freely extractable L-glutamic acid. Incubation of cells with glucose alone leads to a decline in the endogenous glutamate level. In the presence of both glutamic acid and glucose large amounts of L-glutamate are accumulated, generally on the order of 10 per cent of the dry weight of cells used.

In the succeeding studies, accumulation, measured manometrically, was defined as the increase over control levels of intracellular L-glutamic acid. Since assays were performed on extracts of unwashed packed cells, the amount of glutamic acid adhering to cells and trapped in the intercellular space was corrected for by subtracting the amount of L-glutamate found in cells suspended in buffer and glutamate at 2° and centrifuged immediately. Accumulation of isotope was corrected with the use of the same control. Fig. 1*B* shows that the intracellular accumulation of isotope corresponds closely to the accumulation of L-glutamate. In keeping with the results of the manometric assay, there was no accumulation of isotope in the cold and only a very small accumulation in the absence of glucose. Radioautograms prepared from two-dimensional paper chromatograms of cell extracts showed that the isotope was confined virtually exclusively to the region of the paper occupied by glutamic acid. However the isotopic method shows the entrance of slightly more glutamate than is measured manometrically as a net increase over control levels. Relative to the amount taken up, the greatest difference is encountered early in the incubation at which time the discrepancy can be partly accounted for as an exchange of intra- and extracellular glutamate (see Table II). There is also at this time a small conversion of L-glutamate to a substance which is resistant to enzymatic decarboxylation and which has the chromatographic properties of glutamic acid. The amount of isotope in this fraction, which is referred to tentatively as D-glutamic acid, increases during the incubation and at 90 minutes generally accounts for 10 per cent of the isotope present in the cell.

The relation between the initial rate of accumulation and the external glutamate concentration is described in Fig. 2. The rate increases sharply as the concentration is raised, but above 3.0 mM further increases in the external concentration produce relatively small increases in the rate. The curve has the form of an adsorption isotherm, and the data yield a straight line

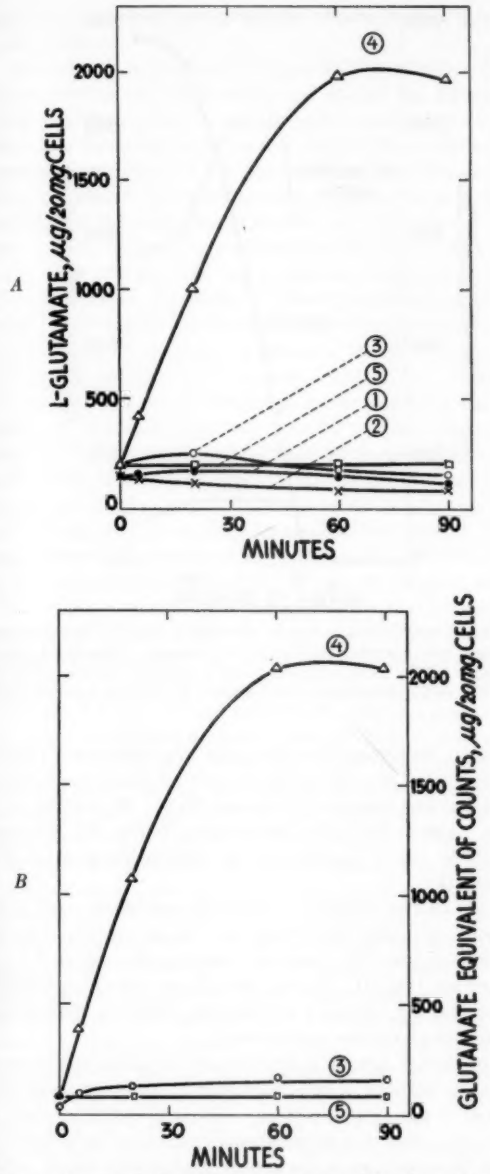

FIG. 1. Accumulation of freely-extractable glutamic acid by *L. arabinosus*. *A*, L-Glutamic acid in ethanol extracts assayed by enzymatic decarboxylation. *B*, Glutamate equivalent of radioactivity in cell extracts. Cells were incubated under the standard uptake conditions modified as follows: *Curve 1*, ●——● without glucose and glutamic acid; *Curve 2*, ×——× without glutamic acid; *Curve 3*, ○——○ without glucose; *Curve 4*, △——△ no omissions; *Curve 5*, □——□ as *4*, incubated at 2°.

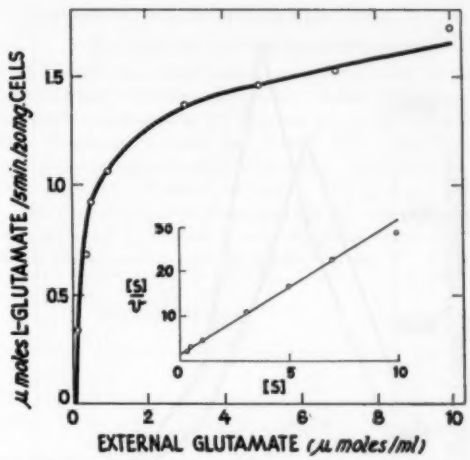

FIG. 2. The effect of external glutamate concentration on the rate of glutamate accumulation by *L. arabinosus*. Standard uptake conditions. The inset shows these data plotted according to the method of Lineweaver and Burk (13).

(Fig. 2, insert) when plotted according to the method of Lineweaver and Burk (13).

The total amount of glutamate accumulated during incubation for 60 minutes also increased as the external glutamate concentration was raised from 0.1 mM to 3.0 mM. Further increases above this external concentration did not materially change the amount of glutamate accumulated. The manometric and isotopic assays agreed closely in all cases. From measurements of the volume occupied by a given weight of cells, and estimates of the intercellular volume and intracellular water space, it can be calculated that at 1.0 mM the apparent intracellular glutamic acid concentration exceeded the extracellular concentration 360-fold and at 0.3 mM 390-fold. At these lower concentrations (0.1 to 1.0 mM) the cells accumulated 75 to 60 per cent of the glutamic acid originally present in the external buffer, while at higher concentrations (3.0 to 10.0 mM) this value fell to 30 to 10 per cent.

Therefore, the accumulation of glutamate by this organism occurs in opposition to large, apparent concentration gradients through the operation of a process characterized by a rate-limiting substrate concentration. The relatively insignificant accumulation which is observed in the absence of glucose or when incubation is carried out in the cold, suggests that accumulation is dependent upon cellular metabolism. In all these respects, the process in *L. arabinosus* is similar to that originally described in bacteria by Gale (2, 3). The nature of this process and its relation to the active transport phenomenon cannot be described with certainty at present, since the intracellular state (free or bound) of the accumulated molecules is unknown.

Effect of pH—Fig. 3 describes the relation between pH and accumulation capacity. Maximal accumulation was achieved with phosphate buffer at an initial pH of 6.5. Maleate at pH 6.5 has been found to be equally effective. The initial accumulation rate was slightly higher in phosphate at pH 6.0, but the amount of amino acid finally accumulated was submaximal as a result of the poor buffering capacity of phosphate in this range.

Incubation at pH values below 6 is unfavorable not only to accumulation, but also to the retention of accumulated glutamate. This was observed in early experiments with the use of the buffered salts solution described by Gale (2), and employing cells at 1.6 mg. per ml. Under these conditions glutamate accumulation stopped abruptly after incubation for 30 to 40 minutes. Subsequently L-glutamate (together with D-glutamate) was released to the supernatant buffer, so that at 90 minutes the cells contained only 40 per cent of the isotope and L-glutamate which had been present at 30 minutes. After incubation for 60 minutes, the buffer had a pH of 5.2 and at 90 minutes a pH of

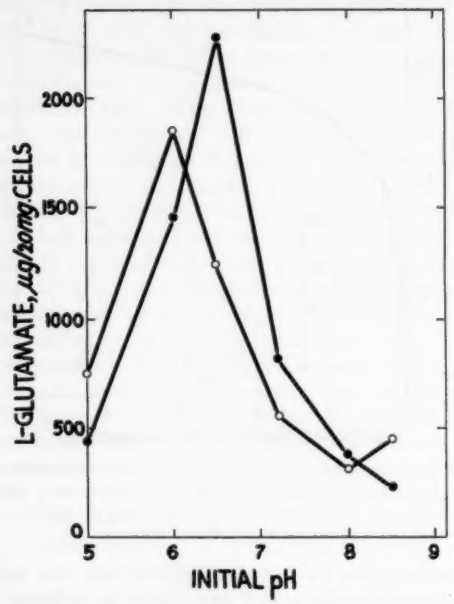

FIG. 3. The effect of pH on L-glutamate accumulation. Standard uptake conditions except for 0.12 M buffers as follows: pH 5.0, acetate; pH 6.0–7.2, phosphate; pH 8.0–8.5 Tris(hydroxymethyl)-aminomethane. The suspensions were incubated for: ○——○, 30 minutes; ●——●, 60 minutes.

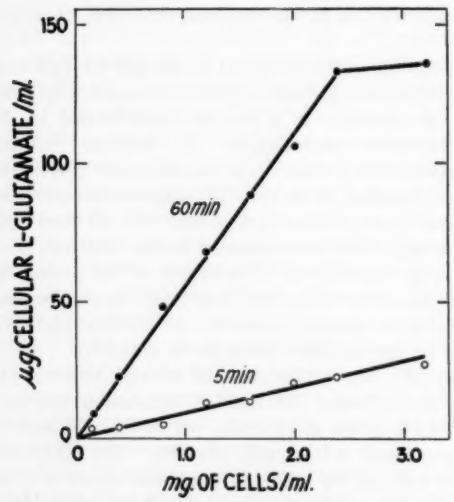

FIG. 4. Effect of cell concentration on the amount of glutamate accumulated by *L. arabinosus*. Washed cells were incubated for the times shown under the standard uptake conditions. L-Glutamic acid in ethanolic cell extracts was determined by enzymatic decarboxylation. The amounts shown have been corrected for zero time values and refer to the intracellular glutamate per ml. of uptake medium.

4.7. A significant metabolism of glutamic acid occurred under these conditions, evidenced by the intracellular accumulation of smaller amounts of L-glutamate than that predicted from the amount of isotope present, by the intracellular accumulation of radioactive γ-aminobutyric acid and by the return to the buffer of a large amount of isotope without an equivalent rise in L-glutamic acid. Increasing the phosphate concentration to 0.12 M largely prevented the pH variation, and maximal accumulation was achieved and maintained between 60 and 90 minutes of incubation. Use of the more concentrated buffer also decreased the metabolic loss of L-glutamate by eliminating glutamic acid decarboxylation.

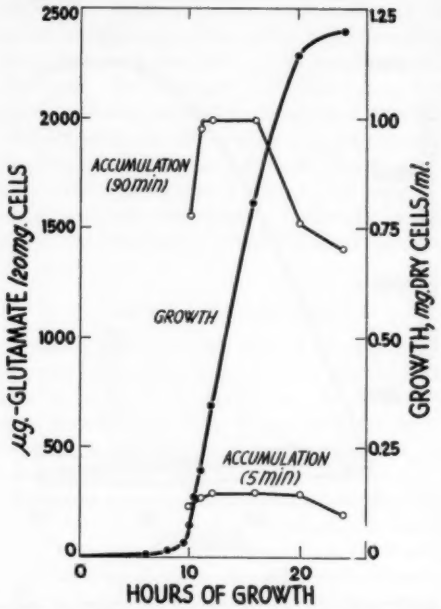

FIG. 5. Effect of culture age on the initial rate and total amount of glutamic acid accumulated by *L. arabinosus*. Standard uptake conditions. Cells were extracted with cold perchloric acid and uptake was calculated from the amount of isotope accumulated.

Effect of Cell Concentration—Under the conditions used in the experiments described here there is a linear relation between L-glutamate accumulated and cell concentration up to 2.4 mg. of cells per ml. (Fig. 4). Hence, the cell concentration normally employed (1.6 mg. per ml.) is a limiting factor in determining the amount of glutamate accumulated.

Effect of Culture Age—Fig. 5 shows the variation in glutamate accumulation activity of cells harvested at different times after inoculation of the growth culture. Maximal activities (rate at 5 minutes and amount at 90 minutes of incubation) were observed with cells from cultures in the exponential growth phase. Cells from cultures entering the phase of maximal growth rate or from cultures no longer increasing in mass showed 70 to 80 per cent of maximal activity. In a few experiments, cells from very young cultures ($\leqq$ 0.1 mg. of cells per ml. at harvest), had a decidedly low capacity (50 per cent of maximum). This variability may be related to the degree of damage sustained during harvesting and washing by these cells which are known to be especially sensitive to physical damage at this phase of the growth cycle. With *L. arabinosus* the initial rate of accumulation was found to be relatively constant and not materially depressed even in those batches of very young cells which exhibited relatively poor accumulation capacity. These results differ from those obtained with *S. aureus* by Gale and Folkes (14) who observed little change in capacity, but a marked variation in accumulation rate as a function of culture age. Such differences may arise principally in seemingly inconsequential variations in methods of cell prep-

aration, particularly, as mentioned above, with cells from very young cultures.

Retention of Accumulated Glutamate—The ability of cells to retain accumulated glutamate was studied as follows. Cells were permitted to accumulate the amino acid for 60 minutes and were then centrifuged and washed once with cold buffer. They were resuspended at 37° in the solutions described in Table I. After additional incubation for 60 minutes the cells were separated from the medium and extracts were prepared in the usual way. Radioactivity was measured in the supernatant solutions and in the cell extracts and L-glutamic acid was measured in the cell extracts. As shown in Table I, incubation at 2° in water, saline, or phosphate led to negligible loss to the external medium of previously accumulated glutamate. A loss of 20 per cent of the isotope during incubation in water at 37° was reduced to 10 per cent or less by incubation in saline or phosphate. In the latter, the presence of glucose tripled the loss as judged by the loss of isotope from the cell. Manometric assays for L-glutamic acid (Table II) showed, however, that in the absence of glucose, despite a superior retention of isotope, there was a greater loss of L-glutamate than in cells incubated with glucose. Thus, in the absence of glucose there was a metabolic loss with a retention in the cell of most of the products. In the presence of glucose, the decline of intracellular L-glutamic acid which was smaller corresponded more closely to the loss of isotope from the cell and to the appearance of isotope in the buffer, and very likely represents a true displacement of the amino acid. Under any of these conditions, the losses were not large and especially in the cold the accumulated glutamate resisted elution from the cell.

The data of Table II show that loss of radioactivity from the cells was greatly increased by the presence in the external buffer of nonradioactive L-glutamic or L-aspartic acids. In the former case, there was a rise rather than a decline in the amount of L-glutamate in the cell indicating a replacement of intracellular (radioactive) by extracellular (nonradioactive) glutamate. Glucose enhanced the glutamate exchange as would be expected if the exchange involved the pathway utilized to accumulate glutamate in the initial incubation. If this interpretation is correct, the large exchange observed with L-glutamate in the absence of glucose might be supported by carbohydrate stores retained from the initial incubation. With aspartic acid the amount of L-glutamate in the cell dropped sharply by an amount closely similar to the glutamate equivalent of the radioactivity appearing in the buffer and lost from the cell. The ability of aspartic acid to elute glutamate from the cell suggests that interaction in the uptake process may account, at least in part, for the lag in the growth response to glutamic acid produced by aspartic acid with this organism (15, 16).

D-Glutamic acid was the least active of the effective displacing compounds. It was almost completely ineffective in displacing isotope in the absence of glucose, and in its presence showed a low level of activity which was not enhanced by varying its concentration between 0.001 and 0.01 M. With the exception of alanine which was slightly active, the other amino acids tested were ineffective in displacing isotope from the cell, testifying to the structural specificity of the displacement phenomenon. However, in the presence of glucose these substances did decrease the amount of L-glutamate (manometric assay) retained in the cell in comparison to the appropriate glucose-incubated control. This enhanced loss of L-glutamate was not observed in the absence of glucose suggesting a requirement for energy, possibly to promote the entry of these amino acids into the cell. Since these amino acids did not stimulate a significant loss of isotope from the cell, the enhanced disappearance of L-glutamic acid probably represents a metabolic conversion of this amino acid to a product which is retained intracellularly. This amino acid-dependent metabolic loss is reminiscent of the observation of Gale and Van Halteren (17) who observed peptide formation

TABLE I

Retention of accumulated glutamic acid

Washed cells of *L. arabinosus* were incubated for 60 minutes under standard uptake conditions. After centrifugation, the cells were resuspended at 1.6 mg. per ml. in the solutions shown below. Aliquots of each suspension were centrifuged immediately (zero time) and after 60 minutes incubation in the resuspension solution. Ethanol extracts of the cells and the supernatant reincubation solution were assayed for radioactivity. Losses of accumulated glutamate were calculated from the difference between the zero time and 60 minute values.

Reincubation solution	Temperature	Loss from cell of isotope
	°C.	%
Water	2	3
Water	37	21
NaCl (0.1 M)	2	2
NaCl (0.1 M)	37	10
Phosphate (0.12 M) + uptake salts	2	4
Phosphate (0.12 M) + uptake salts	37	7
Phosphate (0.12 M) + uptake salts + glucose (0.028 M)	37	23

TABLE II

Elution of accumulated glutamate by amino acids

Conditions of the initial incubation were those described in Table I. Following centrifugation the cells were washed briefly with cold 0.12 M phosphate-salts solution, resuspended at 1.6 mg. per ml. in the solutions described below, and incubated at 37° for 60 minutes. At the time of resuspension 20 mg. of cells contained 1480 μg. of glutamate, determined by the isotopic assay, and 1310 μg. of L-glutamate, determined by the manometric assay.

Addition to phosphate (0.12 M)-salts solution	Decline in glutamate content of cells			
	Without glucose		With glucose	
	Isotope assay	Manometric assay	Isotope assay	Manometric assay
	μg./20 mg. cells			
None	50	400	200	270
L-Glutamic acid (0.003 M)	510	270	820	+310*
L-Aspartic acid (0.003 M)	415		840	840
L-Lysine (0.01 M)	60	430	220	520
L-Alanine (0.01 M)	30	460	280	645
L-Phenylalanine (0.01 M)	0		50	580
D-Glutamic acid (0.005 M)	90		440	585

* The L-glutamate content of these cells increased by 310 μg. per 20 mg.

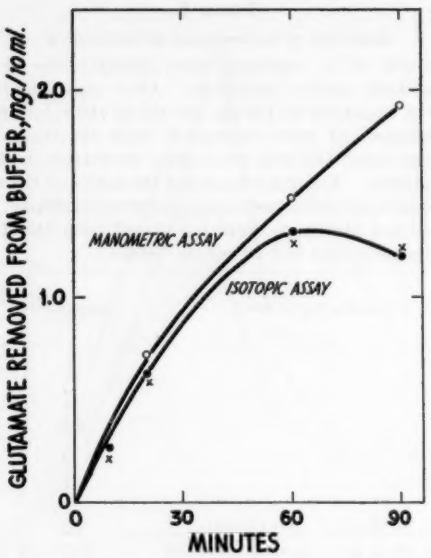

FIG. 6. Comparative removal of isotope and L-glutamate from external buffer during accumulation of glutamate by *L. arabinosus*. Standard uptake conditions. O——O, estimation of L-glutamate uptake by assay of supernatant buffer with glutamic acid decarboxylase; ●——●, estimation of glutamate uptake by determination of residual isotope in supernatant buffer; ×, shows the glutamate equivalent of the isotope found in the respective cell extracts.

TABLE III

Appearance of "D-glutamic acid" in cells during glutamate accumulation by L. arabinosus

Cells from a single culture were harvested at the indicated phases of the growth cycle. After washing, they were incubated for the times shown under standard uptake conditions. Cell extracts were assayed for L-glutamate by enzymatic decarboxylation. The supernatants from this determination were chromatographed in phenol-ammonia-water on sheets of Whatman No. 4 filter paper. Samples for analysis and solutions containing known amounts of glutamic acid were spotted alternately on all sheets. Glutamic acid occurred as a distinct spot well separated from other amino acids. After spraying with ninhydrin the material at the glutamate region was eluted with water, and the color measured. The radioactivity in these samples was determined by eluting exhaustively a duplicate spot not sprayed with ninhydrin, concentrating the eluate to a small volume and plating aliquots for counting. The values shown were corrected for zero time values.

Experiment	Culture density at harvest	Uptake incubation time	Glutamate in cell extracts		
			L-isomer	"D-isomer"	
			Manometric assay	Ninhydrin assay	Isotope assay
	mg./ml.	*min.*	*μg./20 mg. cells*		
1	0.20 (early log)	5	225	150	50
		60	2000	700	380
		90	2500	1110	615
2	0.70 (late log)	5	255	95	80
		60	1855	310	185
		90	2480	100	130

when *S. aureus* was incubated with single amino acids in addition to glutamic acid.

Metabolism of Glutamate during Accumulation—As shown in Fig. 1, the amount of L-glutamate accumulated intracellularly corresponded closely to the glutamate equivalent of the isotope accumulated. In addition, the isotope was found exclusively in the region occupied by glutamic acid in two-dimensional chromatcgrams. Therefore, with the exception of the formation of small amounts of a substance believed to be D-glutamic acid, apparently only a small metabolic loss of L-glutamate occurred during these experiments. Fig. 6 shows, however, that while the loss of isotope from the buffer is accounted for almost entirely by its appearance in the cell extract (there is only a minor incorporation of isotope into the ethanol-insoluble fraction of the cell which accounts for the small difference in these values), the amount of L-glutamate removed from the buffer is considerably higher than that predicted by the amount of isotope removed. This difference is not attributable to the presence in the buffer of glutamic acid decarboxylase inhibitors which would cause an underestimation of L-glutamic acid, and thus indicates the presence of glutamic acid metabolites. Chromatographic studies of the supernatant buffer have demonstrated the absence of isotopically labeled γ-aminobutyric acid or glutamine. D-Glutamic acid is found in the buffer, but in insufficient amounts to account for the major portion of the missing L-glutamic acid.

Therefore, there is a significant metabolic loss of glutamate during these experiments. Since this loss does not occur in the absence of glucose unless the glutamate has already been accumulated (See Table II), it occurs very likely after or during entry into the cell. A metabolic loss of glutamate was shown by Gale (2) and Gale and Mitchell (18) to occur during the accumulation of this amino acid by a strain of *S. faecalis*. These authors did not report the location of the resultant metabolites, but suggested that their formation did not involve peptide synthesis, deamination, or transamination.

Under the conditions used in most of the accumulation experiments described here the only detectable metabolite which accumulates in the cell is a substance believed to be D-glutamic acid. The formation of this material was studied as described in Table III which shows its appearance in cells during glutamate accumulation.[1] The cells used in most of the experiments reported here accumulated approximately 10 per cent of the isotope in the D-glutamic acid fraction (Experiment 2). The amount of D-glutamate found with the use of the colorimetric assay agreed well with the isotopic assay. A variability in the amount of D-glutamate formed during such experiments was traced to the age of the culture used. As shown in Experiment 1 of Table III, the amount of D-glutamate formed is much higher in cells from early exponential phase cultures than in cells harvested at subsequent growth phases. In addition, cells from such low density cultures frequently, although not in every case, accumulated more total D-glutamate than that predicted by the amount of isotope found in this fraction. This suggests that nonradioactive D-glutamic acid, or a substance with closely similar chromatographic properties, is liberated in such cells in a freely ex-

[1] This material has been isolated from streak chromatograms and treated with dinitrofluorobenzene. Only a single derivative was observed whose chromatographic migration in three solvents was found to be identical to that of authentic dinitrophenyl-glutamate. The authors are indebted to Dr. G. Rouser for details of the chromatographic procedures prior to their publication.

tractable form during some accumulation experiments. These observations may have some relation to the biosynthesis of the bacterial cell wall, which is believed to be incomplete in cells from cultures in the early growth phases (19), and which is known to contain D-glutamic acid (20).

Glutamate Accumulation by Other Organisms—The characteristics of glutamate accumulation in *S. faecalis* R and *L. mesenteroides* P-60 have been studied with the techniques described above. Although these organisms accumulate large amounts of L-glutamate, they achieve apparent gradients somewhat smaller than those observed with *L. arabinosus*. As in the latter organism, the uptake is temperature-dependent and does not occur to a significant extent in the absence of glucose.

SUMMARY

Washed cells of *Lactobacillus arabinosus* 17-5 incubated in phosphate buffer containing L-C^{14}-glutamic acid and glucose accumulated this amino acid in a freely-extractable form. Accumulation was negligible in the absence of glucose and at 2°. Under the conditions used, almost all the intracellular isotope was retained in L-glutamic acid. The amount of glutamate accumulated generally equaled, and in some cases exceeded, 10 per cent of the dry weight of cells used. Accumulation occurred in opposition to large apparent concentration gradients, and was characterized by a rate-limiting extracellular amino acid concentration.

Maximal accumulation capacity was observed at pH values between 6.0 and 6.5. Capacity varied with culture age, and was maximal in cells from midexponential phase cultures. Little elution of accumulated glutamate occurred during incubation in water or buffer. However, aspartic and glutamic acids eluted previously accumulated glutamate from the cell.

The metabolic loss of L-glutamate was enhanced when cells were incubated with glucose and alanine, phenylalanine, or lysine. More L-glutamate, but not isotope, was removed from the external buffer than could be accounted for by intracellular accumulation. Approximately 10 per cent of the intracellular isotope was found in a fraction tentatively identified as D-glutamic acid. The amount of this substance detected in cell extracts varied markedly with the growth phase at harvest.

The accumulation of L-glutamate by *Streptococcus faecalis* R and *Leuconostoc mesenteroides* P-60 also has been demonstrated.

Acknowledgment—The authors are deeply indebted to Dr. Eugene Roberts for many stimulating and constructive suggestions.

REFERENCES

1. Holden, J. T., and Roberts, E., *Federation Proc.*, **15**, 276 (1956).
2. Gale, E. F., *J. Gen. Microbiol.*, **1**, 53 (1947).
3. Gale, E. F., *Advances in Protein Chem.*, **8**, 285 (1953).
4. Cohen, G. N., and Rickenberg, H. V., *Ann. inst. Pasteur*, **91**, 693 (1956).
5. Britten, R. J., Roberts, R. B., and French, E. F., *Proc. Natl. Acad. Sci. U. S.*, **41**, 863 (1955).
6. Rickenberg, H. V., Cohen, G. N., Buttin, G., and Monod, J., *Ann. inst. Pasteur*, **91**, 829 (1956).
7. Mitchell, P., and Moyle, J. M., *J. Gen. Microbiol.*, **9**, 257 (1953).
8. Christensen, H. N., Riggs, T. R., and Coyne, B. A., *J. Biol. Chem.*, **209**, 413 (1954).
9. Holden, J. T., Furman, C., and Snell, E. E., *J. Biol. Chem.*, **178**, 789 (1949).
10. Holden, J. T., *J. Biol. Chem.*, **234**, 872 (1959).
11. Holden, J. T., Wildman, R. B., and Snell, E. E., *J. Biol. Chem.*, **191**, 559 (1951).
12. Ayengar, P., Roberts, E., and Ramasarma, G. B., *J. Biol. Chem.*, **193**, 781 (1951).
13. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
14. Gale, E. F., and Folkes, J. P., *Biochem. J.*, **53**, 483 (1953).
15. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157**, 265 (1945).
16. Brickson, W. L., Henderson, L. M., Solhjell, I., and Elvehjem, C. A., *J. Biol. Chem.*, **176**, 517 (1948).
17. Gale, E. F., and Van Halteren, M. B., *Biochem. J.*, **50**, 34 (1951).
18. Gale, E. F., and Mitchell, P. D., *J. Gen. Microbiol.*, **1**, 299 (1947).
19. Shockman, G. D., Kolb, J. J., and Toennies, G., *J. Biol. Chem.*, **230**, 961 (1958).
20. Ikawa, M., and Snell, E. E., *Biochim. et Biophys. Acta*, **19**, 576 (1956).

The Effect of Vitamin B_6 Deficiency on Glutamic Acid Accumulation by *Lactobacillus arabinosus**

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Bacteria have been observed to accumulate amino acids (3–5) and galactosides (6) in opposition to apparent concentration gradients. The mechanism of these accumulations is unknown. A number of investigators (7–9) have suggested that this process may be related to the active transport phenomenon. Riggs *et al.* (10) and Christensen *et al.* (11) have proposed that vitamin B_6 may function as a carrier substance in amino acid transport in mouse Ehrlich ascites tumor cells. It was of interest, therefore, to determine whether vitamin B_6 would influence the activity of a bacterial amino acid accumulation system. Accordingly, a study was undertaken of the effect of vitamin B_6 deficiency on amino acid accumulation in the lactic acid bacteria. This group of organisms was selected for study because of the ease with which the intracellular vitamin B_6 content could be varied (12). It is possible to grow cells of some species containing only minute traces of the vitamin. The activity of a vitamin B_6-dependent amino acid accumulation system would be expected to be reduced in such severely deficient cells. The lactic acid bacteria have been used extensively in analogous experiments to demonstrate the vitamin B_6 dependence of various enzymes (13–15). The general characteristics of glutamic acid accumulation in several lactic acid bacteria have been described (16). This report describes the effect of a vitamin B_6 deficiency on the glutamate accumulation system of *Lactobacillus arabinosus*.

EXPERIMENTAL

Organisms and Media—The organisms, growth media, and methods of cultivation have been described (16). Comparative measurements of accumulation ability were performed with cells grown in aliquots of the same batch of medium which differed only in their content of the vitamin. Inocula for all cultures were grown in the complete absence of vitamin B_6.

L. arabinosus 17-5 was used in most of the experiments. Although this organism is known to synthesize vitamin B_6 on extended incubation in semisynthetic media (12, 17), its growth rate in completely synthetic media lacking the vitamin and containing D-alanine is considerably less than maximal (15). Cultures supplemented with an excess of vitamin B_6 customarily achieve from half to near maximal cell densities within 10 to 12 hours of incubation at 30°. Such cells (HB_6)[1] assayed by previously described methods (12) contain 50 to 200 mμg. of vitamin B_6 per mg. (dry weight[2]) of cells. During such short incubation periods the growth of cultures lacking the vitamin is relatively poor (10 to 15 per cent of maximal densities), and at 12 hours is no longer exponential. These cells (LB_6) contain only 0.005 to 0.010 mμg. of vitamin B_6 per mg. of cells, amounts in the range of the lowest values reported in a previous study (12). Previous experiments (15) also have demonstrated that a variety of transaminases in these cells are at least partially desaturated. All comparative studies of the initial rate of accumulation were performed with cells harvested from media completely lacking the vitamin. In subsequent studies of the total accumulation capacity, cells from cultures supplemented with traces of pyridoxamine (0.005 to 0.020 mμg. per ml.) were used frequently and found to be almost indistinguishable from LB_6 cells in the accumulation experiments. These cells ($LB_6(+)$) contained 0.01 to 0.04 mμg. of vitamin B_6 per mg. of cells.

Cells were washed with cold water before use in accumulation experiments. Suspensions of washed LB_6 and $LB_6(+)$ cells usually contained considerable black debris and clumped cells, which were removed by filtration through glass wool before final adjustment of the suspension density. It should be noted also that LB_6 cells could not be harvested in a Sharples centrifuge. Such cells, shown in another study (18) to be unusually fragile, are probably damaged by this procedure, since cells harvested in this manner have been found repeatedly to have abnormally poor accumulation activity.

The analytical methods and incubation conditions used in accumulation experiments have been described (16).

RESULTS

Described in Fig. 1 is the time-course of accumulation of freely extractable glutamic acid by cells of *L. arabinosus* grown in the complete absence or in the presence of an excess of pyridoxamine. Accumulation was measured in terms of isotope accumulation and as increments in L-glutamate content of the cells. As indicated previously (16), most of the isotope present in the cells can be accounted for as unchanged L-glutamic acid. As shown in Fig. 1, this is true as well for cells depleted of vitamin B_6 except after extended incubation. Most of the discrepancy in the two methods of analysis can be accounted for as a conversion of L-glutamate to a substance with the enzymatic and chroma-

* Preliminary reports of some of this work have appeared (1, 2). This study was supported by a grant (E-1487) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States Public Health Service.

[1] The abbreviations used are: HB_6 cells, high vitamin B_6 cells grown in media supplemented with an excess of the vitamin; LB_6 cells, low vitamin B_6 cells grown in media to which no vitamin B_6 was added; $LB_6(+)$ cells, low vitamin B_6 cells grown in media supplemented with trace amounts of the vitamin.

[2] All descriptions of cell weights refer to the dry weight.

tographic properties of D-glutamic acid. Radioautograms of two-dimensional chromatograms of LB_6 cell extracts showed that the isotope was confined virtually exclusively to the region occupied by glutamic acid. Since, at 5 minutes, only a small amount of isotope was found in substances other than L-glutamic acid, and since this loss was approximately equal in LB_6 and HB_6 cells, the uptake of isotope at 5 minutes was used in most experiments as a rapid and convenient measure of the initial uptake rate.

As shown in Fig. 1, the rate of uptake during the first 5 minutes of incubation was essentially the same in cells grown with and without vitamin B_6. The capacity, indicated by the total amount of free glutamate accumulated during incubation for 60 to 90 minutes, was reduced markedly in cells cultured in the absence of the vitamin. This experiment has been performed with 7 pairs of cultures grown in the absence and in the presence of excess vitamin B_6. In some of these, samples were taken every two minutes during the first 10 minutes of incubation to obtain a more accurate appraisal of the initial accumulation rate. Millipore filters were used in such cases to achieve a rapid separation of cells and buffer. In this series, the rates within a pair agreed to 15 per cent of the value obtained with HB_6 cells. The average initial rates observed in these experiments were as follows: HB_6 cells, 245 ± 39 μg. of glutamate[3] per 5 minutes per 20 mg. of cells; LB_6 cells, 257 ± 25 μg. of glutamate per 5 minutes per 20 mg. of cells. There was a greater variability in the initial rate between experiments than between the pairs of cultures of a given experiment. In an effort to intensify the vitamin B_6 deficiency, LB_6 cells were treated with isonicotinic acid hydrazide (.005 M), semicarbazide (.002 M), or hydroxylamine (.002 M) before and during incubation with glutamic acid. There was no decrease in the initial rate of uptake.

After incubation for 5 minutes the apparent internal glutamate concentration was calculated to be 17 times higher than the external concentration. In similar experiments with the use of a lower external glutamate concentration (0.3 mM), the initial rate, while somewhat lower, was again identical in both cell types, and under these conditions the apparent internal concentration at 5 minutes exceeded the external concentration 90-fold. These studies show, therefore, that under the conditions used here, cells of *L. arabinosus* depleted of all but minute traces of vitamin B_6 accumulate glutamic acid in opposition to an apparent concentration gradient at an initial rate comparable to that observed in cells cultivated with an excess of the vitamin.[4] It must be emphasized that the consistent equality of the accumulation rates was observed only after the fragility of LB_6 cells was recognized and appropriate care exercised in cell preparation. It was also essential to avoid incubation of LB_6 cells in growth medium past the period of active growth since this caused marked losses in accumulation activity (rate and capacity).

The absence of an effect of vitamin B_6 deficiency on the initial uptake rate contrasts with the large decline in accumulation capacity observed with deficient cells. Summarized in Table I are the results of a number of comparative experiments in which the accumulation capacity of LB_6, $LB_6(+)$, and HB_6 cells was determined. In general, HB_6 cells accumulated 3 to 5 times as much glutamate as did either deficient types.

Effect of Preincubation with Pyridoxal, Pyridoxal Phosphate, and Inhibitors—The effect of vitamin B_6 deficiency on accumulation capacity could not be reproduced by preincubating HB_6

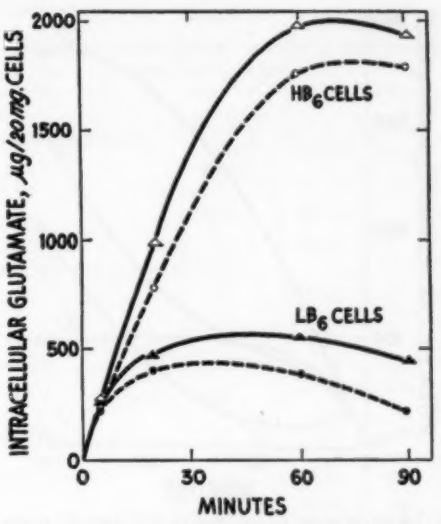

FIG. 1. Accumulation of freely-extractable glutamic acid by *L. arabinosus*; effect of vitamin B_6 deficiency. Cells were harvested after 12 hours growth in medium supplemented either with 1 μg. per ml. of pyridoxamine or with none of the vitamin. Washed cells were incubated under the standard uptake conditions (16). After incubation for the times shown the cells were frozen, thawed and then extracted with ethanol-water. The cell extracts were assayed for radioactivity (——) and L-glutamic acid (----). All values are corrected by an unincubated control. The L-glutamate values also were corrected for a loss of endogenous L-glutamate which was observed in control tubes containing glucose and no L-glutamate. △ and ○, cells grown with pyridoxamine; ▲ and ●, cells grown without pyridoxamine.

TABLE I

Glutamate accumulation capacity of cells grown with and without vitamin B_6

Cell type*	Pyridoxamine concentration in growth medium	No. of experiments	Glutamate accumulation capacity†	
			Average	Range
	μg./ml.		μg./20 mg. cells	
LB_6	None	9	380	230–550
$LB_6(+)$	5 to 20 × 10^{-6}	14	450	280–750
HB_6	1.0	16	1900	1500–2400

* All cultures were harvested after 11 to 13 hours of incubation at 30°.

† Accumulation was measured under standard uptake conditions. The maximal amount of glutamate accumulated during incubation for 90 minutes was tabulated in those cases where vitamin B_6-deficient cells lost glutamate in the terminal phases of incubation. In all cases, the amount of glutamate was calculated from the amount of isotope found in cell extracts. In four experiments with each cell type, accumulation was also estimated by manometric assay of L-glutamic acid.

[3] Standard deviation of the arithmetic mean.

[4] The effectiveness of the deficiency conditions is indicated by the complete or partial inactivation of a variety of vitamin B_6-dependent amino acid decarboxylases and transaminases. The desaturation of the glutamic acid racemizing system also was demonstrated with disrupted cells. However, it should be emphasized that a deficiency state leading to complete inactivation of all known vitamin B_6-dependent enzymes would not constitute proof that an unaffected reaction functions independently of the vitamin.

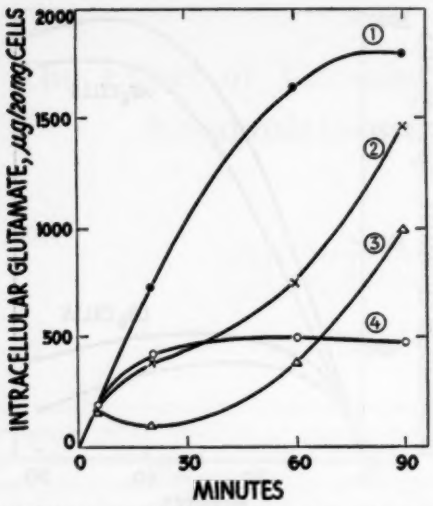

FIG. 2. Effect of nutritional status and growth phase on the time-course of glutamate accumulation. *L. arabinosus* was cultured and harvested under the following conditions: *Curve 1*, complete medium, cells harvested in mid-exponential growth phase at a density of 0.35 mg. per ml.; *Curve 2*, complete medium, cells harvested in early exponential growth phase at a density of 0.20 mg. per ml.; *Curve 3*, low biotin medium (0.045 mμg. per ml.), cells harvested in the period of declining growth rate at a density of 0.18 mg. per ml.; *Curve 4*, low vitamin B_6 medium (0.020 mμg. per ml.), cells harvested in the period of declining growth rate at a density of 0.19 mg. per ml. Water-washed cells were incubated under standard uptake conditions. Frozen cells were extracted with cold perchloric acid, and glutamate uptake was determined from the isotope content of these cell extracts.

cells with isonicotinic acid hydrazide (0.005 M), semicarbazide (0.002 M), or hydroxylamine (0.002 M), or by adding these substances to the uptake system. Under these conditions, there were extensive, vitamin B_6-reversible changes in the intracellular content of free amino acids, indicating the response of various vitamin B_6-dependent enzymes to the pretreatment. Such preincubation was also ineffective on cells grown with low levels of vitamin B_6 just sufficient to permit maximal growth. These cells contain moderately low levels of vitamin B_6 (12) and have a nearly normal accumulation capacity. Furthermore, incubation or preincubation of LB_6 and $LB_6(+)$ cells with various combinations of pyridoxal, pyridoxal phosphate, glucose, and ATP did not improve the accumulation capacity. Under these conditions, the glutamic acid decarboxylase, one of the more readily desaturated enzymes, was reactivated. These results suggest that the effect of the deficiency on accumulation capacity is probably indirect, and not a reflection of the inefficient operation of a vitamin B_6-dependent enzyme functioning directly in the accumulation process.

Glycolytic Activity—A comparison was made of the glycolytic rate in cells grown with and without vitamin B_6. Glycolysis was estimated as carbon dioxide liberation from bicarbonate buffers either under the conditions used to measure accumulation, or under the conditions (complete glycolytic system) described by Clark and MacLeod (19). As normally prepared for uptake experiments (12-hour cultures) LB_6 and HB_6 cells exhibited identical rates of acid production in either test system. Therefore, the reduced accumulation capacity of LB_6 cells cannot be attributed to an inadequacy of this energy-producing system of the cell. This result was unexpected in view of the extensive leakage of cell constituents observed with LB_6 cells (20). The possibility remains that this leakage leads to a defective utilization by LB_6 cells of high-energy compounds produced during glycolysis.

Effect of Other Deficiencies and of Population Density at Harvest—The growth rates and final cell densities of the vitamin B_6-supplemented and -unsupplemented cultures used in these experiments differed considerably. Cultures were harvested routinely after 11 to 13 hours of incubation since preliminary studies (16) showed that maximal accumulation capacity was expressed in such cells. At this time vitamin B_6-supplemented cultures were past the midpoint of the logarithmic growth phase and had attained a culture density of 0.25 to 0.65 mg. per ml.[5] Unsupplemented cultures were near the end of their period of growth and had attained a density of 0.05 to 0.16 mg. per ml. Cells provided with small amounts of the vitamin were also near the end of their period of growth and, depending upon the size of the supplement, had achieved a cell density of 0.10 to 0.32 mg. per ml. Accordingly, the effect of growth rate and cell density of the culture at harvest on the accumulation activity was studied. In some experiments, cultures supplemented with the usual excess of pyridoxamine were harvested at low cell densities. In others, cells were harvested from cultures in which the total amount and rate of growth were reduced to levels comparable with those observed in vitamin B_6-deficient cultures by supplying appropriately small amounts of various amino acids and vitamins.

The results of these experiments are presented in Table II and can be summarized as follows. (*a*) Cells harvested from complete medium in the early stages of the growth cycle have an accumulation capacity only slightly lower than maximum. In all cases this is 2 to 3 times higher than the LB_6 or $LB_6(+)$ cells harvested at the same density. (*b*) Cells from low density cultures in which growth was restricted by limiting the availability of a nutrient were more variable in their behavior. Most of these were slightly less active than HB_6 cells harvested at the same density, but still twice as active as LB_6 and $LB_6(+)$ cells at the same density and phase of growth. In only one case (low pantothenate) was the capacity of such cells as low as that of the $LB_6(+)$ cells harvested at the same density. It can be concluded, therefore, that the observed effect of vitamin B_6 deficiency on accumulation capacity is not attributable solely to the use of cells from low density cultures. On the other hand, the effect is not absolutely specific for this deficiency as evidenced by the behavior of cells from a culture deficient in pantothenic acid.

Studies of the time-course of accumulation revealed, however, that all cell types from control, low density cultures could be distinguished, on the one hand, from HB_6 cells harvested from cultures which had reached at least the midpoint of the exponential growth phase, and on the other, from LB_6 and $LB_6(+)$ cells. Some of these results are presented in Fig. 2. HB_6 cells harvested at densities equal to or greater than 0.35 mg. per ml. accumulated glutamate at a nearly linear rate until the saturation capacity was approached. In contrast, the accumulation rate of all low density cell types declined after approximately 10 minutes of incubation. With LB_6 and $LB_6(+)$ cells, accumulation invariably ceased shortly thereafter. All the other low density cell types were distinguishable from $LB_6(+)$ cells either by a

[5] In all cases, the growth of large volume standing cultures is described. Growth in small aliquots of medium is superior, particularly with the vitamin B_6-deficient cultures.

resumption of accumulation late in the incubation period, in those cases where it had ceased (low biotin), or by a marked acceleration of the rate, in those cases where accumulation was still in progress (HB_6, early exponential phase). The only exception to this correlation was observed with $LB_6(+)$ cells harvested from cultures which had been provided with sufficient vitamin B_6 to attain final cell densities (15 hours) of at least 0.30 mg. per ml., and which were harvested earlier in the growth period, before the appearance of morphological variants (18). However, even these cells showed only a slight tendency to increase uptake late in the incubation period. Cells from pantothenate-deficient cultures behaved like the low-biotin cells, except that the resumption of accumulation occurred later, thus accounting for the lower capacity observed in a 90-minute experiment (Table II). Cells from niacin-limited cultures behaved in a distinctive manner. The initial rate of accumulation by these cells was reduced greatly, and, subsequently, there was a linear accumulation at a still lower rate to the levels shown in Table II. The mechanism by which a vitamin B_6 deficiency prevents accumulation late in the incubation period is under investigation.

D-Glutamate Formation—Freshly harvested cells of *L. arabinosus* contain a freely-extractable substance which resists enzymatic decarboxylation and migrates on paper chromatograms like glutamic acid (16). Approximately equal amounts of this substance, believed to be D-glutamic acid, were found in LB_6 and HB_6 cells. During glutamate accumulation, the appearance of isotope in this D-glutamate fraction in most experiments was comparable in both cell types harvested at equivalent growth phases. While these observations suggested that the deficiency failed to influence the activity of the glutamate racemizing system, experiments with disrupted cells showed that LB_6 cells converted D-glutamate to the L-isomer at only 15 to 20 per cent of the rate observed with HB_6 cells. Preincubation of LB_6 cell preparations with pyridoxal phosphate increased this activity nearly to control HB_6 levels.

Effect of Vitamin B_6 Deficiency in S. faecalis R—The effect of vitamin B_6 deficiency on the glutamate accumulation capacity of *S. faecalis* R has been studied briefly. In a limited number of experiments, a distinct reduction, averaging about 50 per cent, was observed in the total amount of glutamate accumulated by the deficient cells. The deficient cells used were harvested at 12 hours, near the end of the period of exponential growth, at a cell density 40 per cent of the maximum observed in control, vitamin B_6-supplemented cultures. The activities of various transaminases (15) and the alanine racemase (21) are severely restricted in such cells.

TABLE II

Glutamate accumulation capacity of cells from low density cultures

Growth-limiting nutrient	Concentration of nutrient in growth medium	Cell density at harvest*	Growth phase at harvest†	Glutamate accumulation capacity‡
	mμg./ml.	mg./ml.		μg./20 mg.
Pyridoxamine	0	0.07	S	415
Folic acid, *p*-aminobenzoic acid	0	0.08	S	1510
Pyridoxamine	0	0.10	S	440
Pyridoxamine	0.010	0.11	LE	495
None		0.10	EE	1610
Pyridoxamine	0.015	0.15	DG	495
Biotin	0.050	0.15	DG	1250
Pantothenic acid	9.0	0.17	DG	550
Niacin	13.	0.17	LE	900
Leucine	6000	0.15	LE	1190
None		0.17	EE	1410
Pyridoxamine	0.025	0.24	LE	830
Biotin	0.065	0.25	DG	1600
Leucine	6000	0.24	DG	1550
Methionine	2500	0.26	LE	1550
None		0.24	EE	2070
Pyridoxamine	0.050	0.30	LE	860
None		0.31	ME	2120
Pyridoxamine	0.10	0.36	LE	1310
None		0.35	ME	1980

* All cultures were harvested after 10 to 15 hours of incubation at 30°.

† The abbreviations used are: EE, early exponential; ME, midexponential; LE, late exponential; DG, declining growth rate; S, stationary.

‡ The maximal amount of glutamate accumulated during a 90-minute incubation period is shown in those cases where losses occurred in the terminal phase of incubation. The standard uptake conditions were used, and accumulation was determined, in most cases, by the amount of isotope found in cell extracts.

DISCUSSION

A severe vitamin B_6 deficiency in *L. arabinosus* reduces the amount of glutamic acid which this organism can accumulate without diminishing the initial rate of accumulation. The failure of the deficiency to impair the initial rate lends no support to the hypothesis that the amino acid accumulation process in this bacterium contains a vitamin B_6-activated component. Mention should be made, however, of two theoretical limitations which could have prevented its detection in these experiments. These are (*a*) the suboptimal reaction rate of such a system as a result of cofactor depletion would be detected readily only if the vitamin B_6-dependent portion of the accumulation process were normally one of the rate-limiting steps; (*b*) the hypothetical system might have an extremely high affinity for the vitamin which would permit it to retain adequate amounts of the vitamin and thus to function at its maximum rate even under the severe deficiency conditions imposed in this study. The first of these two possibilities cannot be evaluated experimentally at this time; the second cannot be eliminated from consideration, regardless of the extent of desaturation of other vitamin B_6-dependent enzymes, unless one were to obtain cells lacking all traces of the vitamin.

A vitamin B_6 deficiency, however, does reduce markedly the amount of glutamic acid which can be accumulated on extended incubation by *L. arabinosus* and *S. faecalis*. In *L. arabinosus* this reduction in accumulation capacity exceeds the slightly lowered capacity observed in other deficient cell types. Since vitamin B_6, under a variety of conditions, showed no activity in increasing the accumulation capacity of nongrowing cells, it would seem that its role in this process is indirect, *i.e.* that the accumulation of glutamic acid does not depend on the adequate

operation during the actual uptake period of a vitamin B_6-dependent enzyme. Instead, some function relating to the determination of accumulation capacity during the growth of these cells is indicated. It must be recognized that this conclusion rests, to a large extent, on the failure of vitamin B_6 to reactivate deficient cells, and therefore assumes that the deficiency does not cause secondary changes which might prevent reactivation by vitamin B_6 alone. Separate studies (20) have shown that vitamin B_6-deficient cells of *L. arabinosus* are notably incapable of retaining a variety of intracellular constituents. Therefore, if other low molecular weight components, in addition to vitamin B_6, were required for the activity of the system which determines accumulation capacity, the failure to observe reactivation with vitamin B_6 might have been caused by a leakage of such substances from the cell. Indeed, this may explain why vitamin B_6-deficient cells, unlike other vitamin-deficient types, failed to increase accumulation capacity late in the incubation period even when preincubated with or incubated in the presence of vitamin B_6. An investigation of conditions favorable to reactivation of vitamin B_6-deficient cells is in progress.

The effect of a vitamin B_6 deficiency on accumulation capacity, as well as the unusual diphasic uptake seen with most low density cell types might be explained in terms of a heightened instability of a structural component in all such cells which leads to a progressive inactivation of the concentrative mechanism during incubation. A cell wall lacking sufficient rigidity to withstand osmotic stresses could account for such behavior. This suggestion is consistent with reports that cell wall synthesis is incomplete in cells in the early phases of growth (22), and that vitamin B_6-deficient cells of *L. arabinosus* are morphologically abnormal (18).

Much of the evidence bearing on the proposal that vitamin B_6 participates in amino acid transport in mammalian cells has been summarized recently (23). Additional evidence which appears to support this hypothesis has been reported subsequently (24, 25). Further investigation will be required to determine whether the apparently contradictory findings reported here arise from a difference in the mechanism of the bacterial accumulations, from some limitation of the experimental procedure as discussed above, or because the action of the vitamin in the mammalian system is less direct than has been proposed.

SUMMARY

The accumulation of freely-extractable L-C^{14}-glutamic acid was compared in cells of *Lactobacillus arabinosus* 17-5 grown in the absence and presence of vitamin B_6. Despite the depletion of all but trace amounts of intracellular vitamin B_6, deficient cells accumulated glutamate in opposition to an apparent concentration gradient at an initial rate equal to that observed in cells grown with an excess of the vitamin. The initial rate of accumulation in both cell types was unaffected by preincubation with isonicotinic acid hydrazide, semicarbazide and hydroxylamine.

On extended incubation, vitamin B_6-deficient cells accumulated only one-fifth to one-third as much glutamate as did vitamin B_6-adequate cells. However, the accumulation capacity of non-growing vitamin B_6-deficient cells could not be improved by preincubation with various combinations of pyridoxal, pyridoxal phosphate, glucose, and ATP. The presence of these compounds during accumulation also was ineffective. Equal glycolytic rates were observed with vitamin B_6-deficient and -adequate cells.

The glutamate accumulation characteristics of cells cultured with growth-limiting amounts of amino acids and other vitamins also was studied. Cells from all low density cultures had slightly to moderately reduced capacities, but in only one case (low pantothenate) was this as low as that of vitamin B_6-deficient cells. The accumulation rate of all low density cell types declined in the intermediate portion of the incubation period. With the exception of vitamin B_6-deficient cells, this was followed in all cases by an increase in the accumulation rate late in the incubation period, the extent of which largely determined the accumulation capacity.

Vitamin B_6-deficient cells of *Streptococcus faecalis* R also exhibited a reduced capacity for L-glutamic acid accumulation.

The evidence presently available does not indicate a direct participation of vitamin B_6 in the glutamate accumulation process in *Lactobacillus arabinosus*.

Acknowledgments—This study evolved and was undertaken as a result of numerous stimulating discussions with Dr. Eugene Roberts. The work was greatly aided thereafter by his continued enthusiastic support. The technical assistance of Miss Jane Holman is gratefully acknowledged.

REFERENCES

1. Holden, J. T., and Roberts, E., *Federation Proc.*, **15**, 276 (1956).
2. Holden, J. T., and Holman, J., *Federation Proc.*, **16**, 198 (1957).
3. Gale, E. F., *Advances in Protein Chem.*, **8**, 285 (1953).
4. Cohen, G. N., and Rickenberg, H. V., *Ann. inst. Pasteur*, **91**, 693 (1956).
5. Britten, R. J., Roberts, R. B., and French, E. F., *Proc. Natl. Acad. Sci. U. S.*, **41**, 863 (1955).
6. Rickenberg, H. V., Cohen, G. N., Buttin, G., and Monod, J., *Ann. inst. Pasteur*, **91**, 829 (1956).
7. Rowlands, D. A., Gale, E. F., Folkes, J. P., and Marrian, D. H., *Biochem. J.*, **65**, 519 (1957).
8. Mitchell, P., and Moyle, J., in E. T. C. Spooner and B. A. D. Stocker, (Editors), *Bacterial anatomy*, Cambridge University Press, London, 1956, p. 150.
9. Cohen, G. N., and Monod, J., *Bacteriol. Revs.*, **21**, 169 (1957).
10. Riggs, T. R., Coyne, B., and Christensen, H. N., *Biochim. et Biophys. Acta*, **11**, 303 (1953).
11. Christensen, H. N., Riggs, T. R., and Coyne, B. A., *J. Biol. Chem.*, **209**, 413 (1954).
12. Holden, J. T., Furman, C., and Snell, E. E., *J. Biol. Chem.*, **178**, 789 (1949).
13. Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 557 (1944).
14. Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161**, 311 (1945).
15. Holden, J. T., Wildman, R. B., and Snell, E. E., *J. Biol. Chem.*, **191**, 559 (1951).
16. Holden, J. T., and Holman, J., *J. Biol. Chem.*, **234**, 865 (1959).
17. Bohonos, N., Hutchings, B. L., and Peterson, W. H., *J. Bacteriol.*, **44**, 479 (1942).
18. Holden, J. T., and Holman, J., *J. Bacteriol.*, **73**, 592 (1957).
19. Clark, J. A., and MacLeod, R. A., *J. Biol. Chem.*, **211**, 531 (1954).
20. Holden, J. T., *Biochim. et Biophys. Acta*, **29**, 667 (1958).
21. Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, **178**, 799 (1949).
22. Shockman, G. D., Kolb, J. J., and Toennies, G., *J. Biol. Chem.*, **230**, 961 (1958).
23. Moldave, K., *Arch. Biochem. Biophys.*, **75**, 418 (1958).
24. Riggs, T. R., and Walker, L. M., *J. Biol. Chem.*, **233**, 132 (1958).
25. Jacobs, F. A., and Hillman, R. S. L., *J. Biol. Chem.*, **232**, 445 (1958).

The Utilization of Glycine by the Nucleated Erythrocyte*

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The citric acid cycle is usually considered to be the mechanism by which most cells obtain the major portion of their energy. This appears to be true of the nucleated erythrocyte in which the cycle seems to play an additional important role; that of supplying certain essential metabolites.

Porphyrins, intermediates in heme formation, are known to be synthesized in nucleated-red blood cells from two simple precursors (1), glycine and the citric acid cycle member, succinate. Although the α-carbon atom of glycine, but not the carboxyl carbon, was shown to be utilized directly for porphyrin synthesis (2, 3), no conclusive evidence has been presented as to the possible indirect participation of this amino acid in porphyrin formation. Glycine was demonstrated to account for 8 of the 34 carbon atoms of protoporphyrin, while the remaining 26 were shown to be derived from acetate by way of an unsymmetric 4-carbon atom compound (1, 4, 5). This unsymmetric precursor is thought to be succinyl-Coenzyme A (6) arising from the citric acid cycle either from α-ketoglutarate or succinate, or both.

The present work was undertaken to determine whether glycine, like acetate (7), could be metabolized appreciably via the citric acid cycle in nucleated erythrocytes and thus could contribute significantly to the succinyl moiety of the pyrrole rings of porphyrins and perhaps also to the ureido groups of purines or other compounds as was previously postulated (8). Although glycine may not be incorporated into the cycle acids to the same extent as was acetate, the results obtained in this investigation, nevertheless, warrant the conclusion that glycine can contribute ndirectly to the biosynthesis of succinate and α-ketoglutarate.

EXPERIMENTAL

For this investigation, the previously described procedures for the study of the citric acid cycle in erythrocytes (7) were used. For each point under consideration two to three separate runs were performed. Because the metabolic pathways of glycine are numerous and its utilization by certain tissues is extensive, the use of larger amounts than of acetate for incubation with red cells was found necessary. After 50 mg. of the amino acid were added to the erythrocyte preparations, they were incubated for either 4 or 24 hours. In one experiment DPN was added to the incubation mixtures in case this cofactor may have been washed out during processing of cells. When it was found that the added DPN did not increase the levels of the intermediate acids appreciably, it was omitted in subsequent experiments.

Use of Glycine-2-C^{14}—Experiments utilizing glycine-2-C^{14}, similar to those with acetate-1-C^{14} (7), were performed in order to obtain further evidence for the incorporation of this amino acid into the cycle. Glycine-2-C^{14}, 0.5 mg., (activity 1.16 mc. per mmole) was mixed with 50 mg. of the nonlabeled acid. The activity obtained varied from a few counts to 150 c.p.m. over and above the background. As this amount of activity is too low to permit accurate interpretations, the experiments were repeated with a higher concentration of the labeled glycine (1.8 mg. of glycine-2-C^{14} plus 20 mg. of the nonlabeled amino acid).

RESULTS AND DISCUSSION

From the experiments represented in Table I it is clear that glycine can be metabolized by way of the citric acid cycle, giving rise to all of the components of the cycle. It may be noted also that the concentrations of intermediates obtained are almost identical with those resulting from incubation with acetate. That the similarity in the pattern of incorporation is striking indeed, can be seen by comparing the levels of the acids synthesized in the cells after incubation with the two substrates for a 4-hour interval.

With regard to the effect of time of incubation on the incorporation of glycine in the cycle, the data of Table I reveal that there is no great difference between the levels obtained after 4 hours or 24 hours of incubation. However, as might be expected, the amounts are somewhat less in the longer interval.

Experiments with glycine-2-C^{14} are summarized in Table II. It may be noted that the levels of the acids are similar to those obtained when nonlabeled glycine was used as a substrate. Furthermore, the concentrations of the individual acids are of the same magnitude as was found with acetate (7). However, the degree of incorporation of glycine-2-C^{14} appears to be less than that found in experiments employing acetate-1-C^{14}. This is to be expected since the acetate used was much more active than the glycine (4.11 mc. per mmole for acetate and 1.16 mc. per mmole for glycine). Moreover, the isotopic glycine was diluted with nonlabeled carriers to a greater extent. In addition, the place of labeling is different in the two compounds, being in the carboxyl and methyl carbon in acetate and glycine, respectively. The differences of labeling in the substrates would, certainly, result in C^{14} activity at different positions in the molecules of the various cycle acids. Nevertheless, the present results provide unequivocal evidence of the incorporation of glycine into the citric acid cycle. The conclusion appears warranted, therefore, that glycine may contribute both directly (8) and indirectly to the biosyntheses of porphyrins and perhaps of other compounds by way of the tricarboxylic acid cycle in nucleated erythrocytes. This concept is in line with the succinate-glycine cycle (8, 9) which has been postulated in order to explain the formation of δ-aminolevulinic acid, a precursor of porphyrins, as well as some other features of the metabolism of glycine.

* The data in this paper are taken from a dissertation submitted to the Graduate School of Wayne State University by Rashid M. Dajani, in partial fulfillment of the requirements for the degree Doctor of Philosophy, June 1957. Supported by Research Grant No. CY 2144 from the National Institutes of Health. A preliminary report was presented before the American Society of Biological Chemists at Chicago, April 1957.

TABLE I

Average concentration in mmoles per l. × 10^{-3} of Krebs cycle acids in nucleated erythrocytes incubated with glycine*

Acid	Control	Glycine (50.0 mg.)		Glycine + DPN (2.0 mg.)
		4 hours' incubation	24 hours' incubation	4 hours' incubation
Lactic	4.3	6.8	5.6	6.7
Pyruvic	15.4	42.9	41.8	42.2
Citric	0.9†	26.7	26.7	26.7
Aconitic (*cis* and *trans*)	1.1†	1.6	1.5	1.6
Isocitric	1.0†	1.9	1.8	1.9
α-Ketoglutaric	2.4	18.7	18.2	19.0
Succinic	5.1	49.0	47.8	48.2
Fumaric	12.0	57.5	56.0	56.8
Malic	3.2	37.2	36.4	38.8
Oxalacetic	4.1	30.5	29.3	33.1

* Average of two to three separate runs.
† Amount too small to be determined accurately.

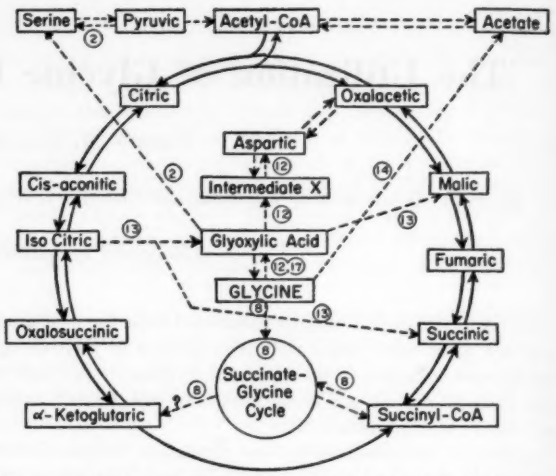

FIG. 1. Postulated pathways for the degradation of glycine by way of the citric acid cycle. The numbers are for references.

Because it has been conclusively established that glycine enters the citric acid cycle as one route of its degradation, the question may be posed as to the mechanism or mechanisms by which this occurs. Several pathways for glycine catabolism have been proposed. Weinhouse (10), in a review of the synthesis and degradation of glycine summarized two of these metabolic channels as follows: The first leads to CO_2 formation by way of glyoxylate → formate, while the second involves the formation of serine → pyruvate → citric acid cycle. However, this investigator presented evidence and reasons which argue against the second as a major pathway for glycine oxidation. In fact, his studies *in vitro* in rat liver suggest that this route represents a quantitatively unimportant pathway of glycine catabolism. Indeed, some evidence in favor of the first route as a major pathway for glycine degradation has been obtained by several workers (10–13). Sprinson (14), on the other hand, has demonstrated that acetic and aspartic acids, precursors to the cycle acids, can be formed from glycine in the rat. The data indicated that both carbon atoms of acetate are derived from the α-carbon atom of glycine. Also the α- and β-carbon atoms of aspartic acid are derived from the methyl carbon of glycine. As a possible mechanism for these transformations he proposed, on purely hypothetical grounds, that glyoxylate, the deamination product of glycine, condenses with glycine to produce a 4-carbon atom intermediate which is in equilibrium with aspartic acid. A similar reaction is described in the microorganism, *Diplococcus glycinophilus*, by Barker *et al.* (15). These authors suggested the condensation of two molecules of glycine, or a derivative of it, at the α-carbon atoms leading to the formation of a C_4-dicarboxylic acid. The carboxyl carbon atoms are converted

TABLE II

Average concentration and specific activities of Krebs cycle acids in nucleated erythrocytes incubated with glycine-2-C^{14} or acetate-1-C^{14}*

Acid	Control	Glycine†	Glycine† + malate‡	Glycine† + malate‡ + fluoroacetate§	Acetate + malate‖	Glycine	Glycine + malate	Glycine + malate + fluoroacetate	Acetate + malate‖
	mmole/l. × 10^{-3}					*c.p.m./mmole × 10^3*			
Lactic	4.28	5.9	8.3	13.2	8.0	0.47	0.47	0.48	19.7
Pyruvic	15.40	43.0	51.0	73.0	45.0	0.48	0.47	0.49	19.4
Citric	0.90¶	4.25	28.75	47.2	28.0	12.8	12.5	12.4	470.0
Aconitic (*cis* and *trans*)	1.1¶	1.5	1.65	0.5¶	1.6	11.3	12.1	12.0	420.0
Isocitric	1.0¶	4.8	5.55	0.6¶	1.6	12.3	12.2	12.4	430.0
α-Ketoglutaric	2.4	17.35	19.50	7.05	16.0	6.7	6.9	6.82	270.0
Succinic	5.1	48.0	48.1	46.5	49.7	7.2	7.3	7.1	332.0
Fumaric	12.0	56.0	57.0	55.5	51.0	6.8	7.0	6.9	278.0
Malic	3.0	36.5	39.0	29.0	39.5	6.91	6.85	6.89	280.0
Oxalacetic	4.05	23.25	27.0	140.0[a]	28.0	7.1	7.2	7.15	280.0

* Average of two to three separate runs.
† Glycine-2-C^{14} (activity 1.16 mc. per mmole), 1.8 mg; glycine, 20.0 mg.
‡ L-Malic acid, 0.05 mg.
§ Fluoroacetic acid, 0.50 mg.
‖ Taken from Table IV, *J. Biol. Chem.*, **231**, 913 (1958) for comparison.
¶ Amount too small to be determined accurately.
[a] Mixed with acetoacetate.

to CO_2, while the remaining carbon atoms are converted into acetic acid. The direct reduction of glycine to acetic acid was shown not to be an important reaction. Still another pathway would be glycine to glyoxylic acid to malic acid by way of the "glyoxylate cycle" of Kornberg and Krebs (16). The accompanying diagram (Fig. 1) summarizes postulated pathways for the degradation of glycine involving the citric acid cycle. Although none of these pathways has been shown to be operative in the nucleated erythrocyte, it would not be unreasonable to postulate that the degradation of glycine may follow one of these routes. Obviously, further studies will be necessary to clarify this point.

In the course of the study with labeled glycine an unknown fraction "*x*" which did not titrate with sodium hydroxide but gave the highest count of all fractions, was eluted from the silica gel column. This fraction assumed a position on the chromatogram between *cis*-aconitic and malic acids. To exclude the possibility that fraction "*x*" was glycine, a 50-mg. sample of the nonlabeled amino acid mixed with 1.0 mg. of the radioactive acid was processed as for erythrocytes. No radioactive fraction could be detected. In another experiment with the use of labeled glycine, the fractions which corresponded to substance "*x*" were pooled, concentrated, and chromatographed on paper. Simultaneously, spots were made from uric acid, guanine, and adenine (substances known to originate, in part at least, from glycine) in addition to pure glycine, in an effort to identify this highly active fraction. This technique indicated that the unknown substance was not any of these compounds. More work is needed for its characterization.

SUMMARY

Glycine was shown to be utilized by the nucleated erythrocytes for the formation of the individual Krebs cycle acids. Similar patterns of the acid spectrum were obtained with glycine-2-C^{14} as with acetate-1-C^{14}. However, there were differences between the amounts and specific activities of the cycle acids when either glycine or acetate was used as a substrate. Nevertheless, the data show that glycine can be used as a precursor for the cycle intermediates and, therefore, can be involved indirectly, as well as directly, in biosyntheses in nucleated erythrocytes.

Mechanisms for the incorporation of glycine into the citric acid cycle are discussed.

REFERENCES

1. Shemin, D., and Wittenberg, J., *J. Biol. Chem.*, **192**, 315 (1951).
2. Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K., *J. Biol. Chem.*, **176**, 319 (1948).
3. Radin, N. S., Rittenberg, D., and Shemin, D., *J. Biol. Chem.*, **184**, 745 (1950).
4. Wittenberg, J., and Shemin, D., *J. Biol. Chem.*, **185**, 103 (1950).
5. Muir, H. M., and Neuberger, A., *Biochem. J.*, **47**, 97 (1950).
6. Shemin, D., and Kumin, S. *J. Biol. Chem.*, **198**, 827 (1952).
7. Dajani, R. M., and Orten, J. M., *J. Biol. Chem.*, **231**, 913 (1958).
8. Shemin, D. Russell, C. S., and Abramsky, T., *J. Biol. Chem.*, **215**, 613 (1955).
9. Nemeth, A. M., Russell, C. S., and Shemin, D., *J. Biol. Chem.*, **229**, 415 (1957).
10. Weinhouse S., in W. D. McElroy and H. B. Glass (Editors), *A symposium on amino acid metabolism*, Johns Hopkins Press, Baltimore, 1955, p. 637.
11. Zelitch, I., and Ochoa, S., *J. Biol. Chem.*, **201**, 707 (1953).
12. Nakada, H. I., and Weinhouse, S., *J. Biol. Chem.*, **204**, 831 (1953).
13. Chance, B., *J. Biol. Chem.*, **182**, 649 (1950).
14. Sprinson, D. B., *J. Biol. Chem.*, **178**, 529 (1949).
15. Barker, H. A., Volcani, B. E., and Cardon, B. P., *J. Biol. Chem.*, **173**, 803 (1948).
16. Kornberg, H. L., and Krebs, H. A., *Nature*, **179**, 988 (1957).
17. Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152**, 119 (1944).

3-Hydroxyanthranilic Acid Metabolism

VII. MECHANISM OF FORMATION OF QUINOLINIC ACID*

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The establishment of 3-hydroxyanthranilic acid as an intermediate in the biosynthesis of nicotinic acid from tryptophan was determined with the use of *Neurospora crassa* as the test organism (1, 2) and in mammals (3). Studies by Heidelberger *et al.* (4) with DL-tryptophan-3-C^{14} demonstrated that kynurenine was one of the compounds in the reaction sequence of tryptophan conversion to nicotinic acid, and that the nicotinic acid isolated was labeled in its carboxyl group. Schayer and Henderson (5) with deutero-N^{15}-indole labeled tryptophan found almost all of the N^{15} incorporated in the pyridine ring of quinolinic acid. Schweigert (6) and Bokman and Schweigert (7) reported that 3-hydroxyanthranilic acid may be quantitatively converted to quinolinic acid using rat liver homogenates or slices. Miyake *et al.* (8) proposed a quinonoid-type structure for the intermediate, but Wiss and Bettendorf (9) have demonstrated that the first intermediate compound in the conversion of 3-hydroxyanthranilic acid to quinolinic acid is an aliphatic cleavage compound, 1-amino-4-formyl-butadiene-1,3-dicarboxylic acid (1, 2). This compound was quantitatively converted to quinolinic acid spontaneously in the absence of any enzymes. Mehler (10), however, showed that 3-hydroxyanthranilic acid oxidase, prepared by protein fractionation of an acetone powder of guinea pig liver, produced picolinic acid and he suggested that 3-hydroxyanthranilic acid oxidase functions in the formation of an aliphatic cleavage product which may then spontaneously form quinolinic acid or may be enzymatically decarboxylated to form picolinic acid.

With the use of a liver enzyme system, Makino *et al.* (11) reported that 3,4-dihydroxyanthranilic acid was as active as HAA[1] in promoting nicotinic acid formation, but this has not been confirmed by other investigators (3, 7, 9). Henderson and Hankes (3) observed that tryptophan labeled in positions 3a, 7a, and 7 formed quinolinic acid labeled in the number two and three positions of the pyridine nucleus, and in the α-carboxyl group. The specific activity of the nicotinic acid subsequently formed was greatly diluted. However, this dilution could be due to loss during the metabolic conversion of quinolinic acid to nicotinic acid or to dilution in the body pool of N'-methylnicotinamide.

The present study was initiated because neither the exact position of cleavage of the molecule in the conversion of HAA to quinolinic acid nor the metabolic fate of carbon atom 3 of HAA has been unequivocally demonstrated. The nature of the reactions involving the carbon atom at position 3 of the HAA ring was considered to be of paramount importance. It was considered that this labeling would demonstrate the mechanism of conversion of HAA to quinolinic acid by tracing the position change of carbon atom 3 in the benzene nucleus of HAA.

A nine-step chemical synthesis starting with phenol-1-C^{14} was designed to prepare HAA exclusively labeled in position 3. In addition to the preparation of the labeled compound, techniques were devised to isolate and purify quinolinic acid, CO_2 liberated, and the nicotinic acid formed. The use of an enzyme system prepared from an acetone powder of rat liver with the use of unlabeled compounds demonstrated the feasibility of this approach.

EXPERIMENTAL

Synthesis of 3-hydroxyanthranilic Acid-3-C^{14}—A solution composed of 100 gm. of phenol-1-C^{14}, I, (stated specific activity 103 c.p.m. per μmole) and 28 ml. of water was nitrated at 15° and the 2-nitro and 4-nitro isomers were separated by indirect steam distillation (12). Total yield was 38.8 gm. of the desired 2-nitrophenol-1-C^{14}, II, (28 per cent),[2] m.p. 44–45°, melting point undepressed on mixture with an authentic sample. II was reduced in acetic anhydride with the use of hydrogen and Raney nickel at 92° (13). The yield of *O,N*-diacetyl-2-aminophenol-1-C^{14}, III, was 34.8 gm. (65 per cent), m.p. 124–125°, reported 124–125°. This diacylated derivative was nitrated at 0° in acetic anhydride and acetic acid to form the 3-nitro (minor product) and 5-nitro isomers, (14). The isomers were separated by their different rates of dissolution in hot 95 per cent ethanol. The total nitration yield was 38.5 gm. (90 per cent); and of 3-nitro-*O,N*-diacetyl-2-aminophenol-1-C^{14}, IV, 13.1 gm. (31 per cent), m.p. 182–183°, reported 183°.

The 3-nitro isomer was then deacylated by heating it for 20 minutes in a wax bath at 200° and then at 235° for an additional 15 minutes (15). Total yield of 2-methyl-4-nitrobenzoxazole-7a-C^{14}, V, was 7.6 gm. (77 per cent), m.p. 124–125°, reported 125°. Reduction of V in ethanolic Raney nickel with hydrogen at 60° for 2 hours produced 5.3 gm. (84 per cent) of 4-amino-2-

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[1] The abbreviation used is: HAA, 3-hydroxyanthranilic acid.

[2] Percentage yields are given on a molar basis from the preceding compound. We are indebted to Dr. Kenneth Kopple, Department of Chemistry, for his counsel in devising the reaction sequence used.

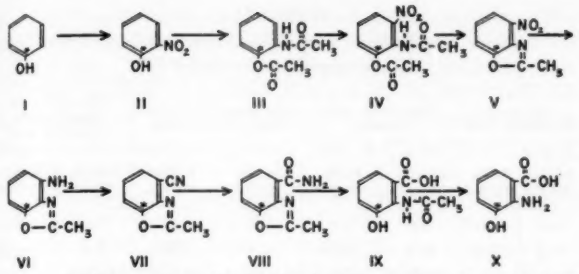

FIG. 1. Chemical synthesis of 3-hydroxyanthranilic acid-3-C^{14} from phenol-1-C^{14}.

methylbenzoxazole-7a-C^{14}, VI, m.p. 78–79°, reported 78–79° (16). This compound was then diazotized with nitrous acid at 5° and the product was added to a cuprous cyanide solution to form 4-cyano-2-methylbenzoxazole-7a-C^{14}, VII, which was purified on an alumina column (16). Total yield was 2.2 gm. (40 per cent), m.p. 108–109°, reported 108–109°.

The following reactions were performed with the method of Sannie and Lapin (16) with slight modifications. VII was hydrolyzed with water in a sealed micro-Carius tube at 180° for 3 hours to form 380 mg. (17 per cent) of 4-carboxamide-2-methylbenzoxazole-7a-C^{14}, VIII, m.p. 170–172°, reported 172°. This derivative was hydrolyzed in 100 per cent orthophosphoric acid with the use of sufficient phosphorus pentoxide, to form *N*-acetyl-3-hydroxyanthranilic acid-3-C^{14}, IX. Total yield was 110 mg. (32 per cent), m.p. 143–144°, reported 143–144°. The acetyl group was hydrolyzed with hot barium hydroxide to form 50 mg. (57 per cent) of the desired compound, 3-hydroxyanthranilic acid-3-C^{14}, X, m.p. 240–250° (decomposes), reported 245–265° (decomposes), melting point undepressed on mixture with an authentic sample. The specific activity of the synthesized HAA was 80 c.p.m. per μmole. Total counts were 2.6×10^4 c.p.m., corresponding to an over-all yield of 0.03 per cent. A summary of the reaction sequence is shown in Fig. 1.

The purity of the HAA was also evaluated spectrophotometrically and photofluorometrically. The ultraviolet spectrum of the product and of an authentic sample were identical (Fig. 2).

Enzyme Preparation—Adult male Holtzman rats were decapitated and the livers removed. An acetone powder was prepared immediately by homogenizing the livers with 20 volumes of cold acetone for 2 minutes and filtering the suspension until dry with the use of suction. The powder was resuspended in 20 parts of acetone and the procedure repeated. The tan powder, 3 gm. of that obtained in this manner, was extracted with 45 ml. of 0.01 M phosphate buffer, pH 7.0, for 45 minutes at 5°, the insoluble matter removed by centrifugation, and the clear supernatant solution used as the enzyme source. In each experiment, the acetone powders were prepared just before use to assure the presence of an adequate amount of active enzyme.

Incubation—Reaction mixtures containing approximately 6 mg. of substrate,[3] 10 ml. of enzyme solution, and sufficient buffer at pH 7.0 to make a final volume of 35 ml. were incubated in open beakers with mechanical stirring for 45 minutes at 37.5°. This procedure is identical to that used in previous studies in this laboratory (7, 8). The reaction was stopped by adding 1 ml. of 20 per cent metaphosphoric acid. The precipitate was removed by filtration and the supernatant solution diluted to 40 ml. for further analysis.

[3] Determinations of HAA by spectrophotometric and photofluorometric methods ranged from 95 to 102 per cent of the amount added.

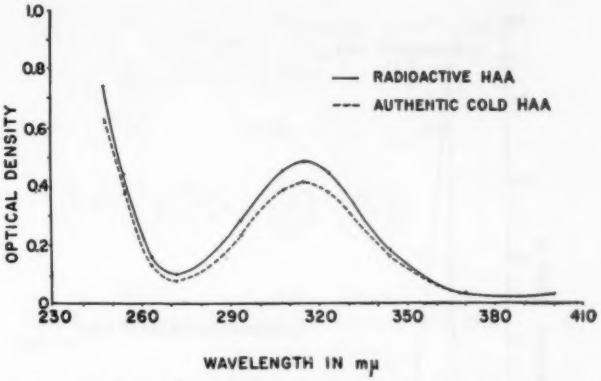

FIG. 2. Ultraviolet absorption spectra of authentic HAA and synthesized radioactive HAA. Concentration of the authentic sample, 25 μg. per ml. and of the synthesized sample 21 μg. per ml.

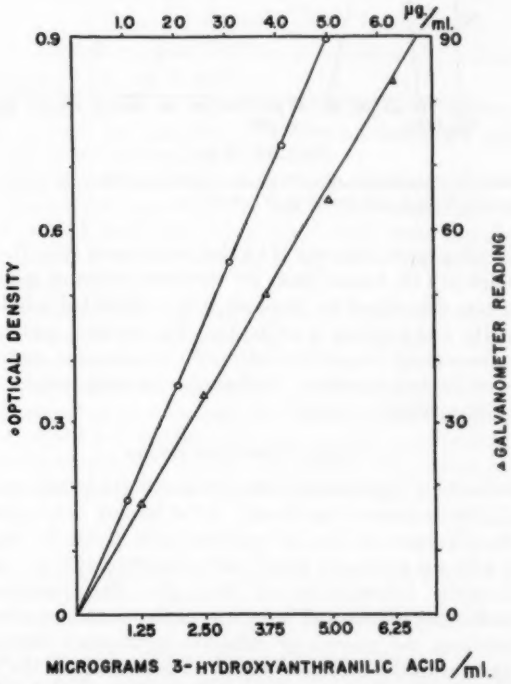

FIG. 3. The optical density, O——O, and fluorescence, △——△, observed with varying concentrations of HAA in phosphate buffer, pH 7.0. The concentrations used in the spectrophotometric determination are shown on the lower abscissa and in the photofluorometric determination are on the upper abscissa.

Analytical Methods—The amount of HAA metabolized was determined fluorometrically as described by Bokman and Schweigert (7) (Fig. 3). The amount of quinolinic acid in the reaction mixture was determined spectrophotometrically in 1-cm. silica cells in a Beckman model DK recording spectrophotometer, with the use of a predetermined molar extinction coefficient of 3610 at 268 mμ.

The quinolinic acid from the incubation mixture was then purified on a Dowex 1 anion exchange column, with 0.1 M $CaCl_2$ as

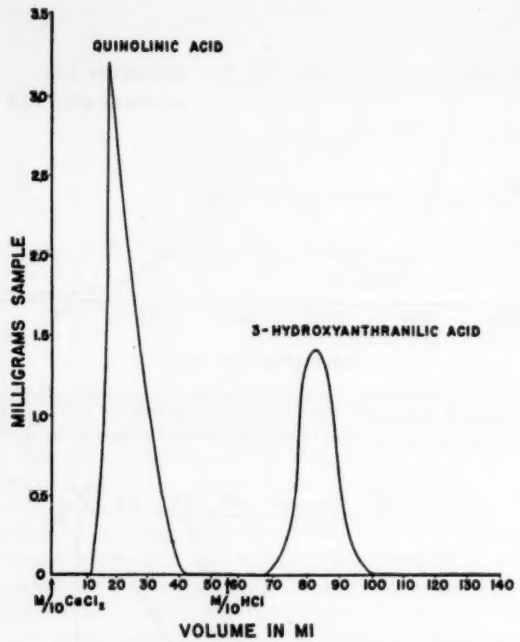

FIG. 4. Separation of quinolinic acid from HAA on a Dowex 1 anion exchange column with 0.1 M HCl.

the eluting agent, from any HAA that was present (Fig. 4). The amount of CO_2 formed from the decarboxylation of quinolinic acid was determined by trapping it in a saturated solution of $Ba(OH)_2$ and weighing it as $BaCO_3$. The nicotinic acid formed was determined microbiologically with *Lactobacillus arabinosus* 17–5 as the test organism. Radioactive counting was done in a gas flow windowless counter.

Enzyme Conversion Studies

Preliminary experiments with nonlabeled HAA indicated the feasibility of determining the fate of the labeled carbon atom-3 in the conversion of HAA to quinolinic acid (Table I). Quinolinic acid was previously found not to interfere with the photofluorometric determination of HAA (7). This permitted a photofluorometric as well as a spectrophotometric method of determining the amount of substrate metabolized during incubation with the enzyme system. Measurements of the HAA

TABLE I

Formation of quinolinic acid, CO_2, and nicotinic acid from nonradioactive HAA

HAA, sample weight	Quinolinic acid formed,* sample weight	CO_2 formed as $BaCO_3$† from quinolinic acid, sample weight	Nicotinic acid formed‡ from quinolinic acid, sample weight
mg.	*mg.*	*mg.*	*mg.*
5.5	5.6 (93%)§	7.3 (103%)	4.2 (95%)
5.1	6.0 (107%)	6.2 (94%)	3.8 (93%)

* Determined spectrophotometrically.

† Determined gravimetrically.

‡ Calculated from a standard curve using *L. arabinosus* as the test organism.

§ Percentage yields calculated on a molar basis.

TABLE II

Yields of quinolinic acid, CO_2, and nicotinic acid from 3-hydroxyanthranilic acid-3-C^{14}

3-Hydroxyanthranilic acid		Quinolinic acid formed*		CO_2 formed as $BaCO_3$† from quinolinic acid		Nicotinic acid formed‡ from quinolinic acid	
Sample weight	Radioactivity	Sample weight	Radioactivity	Sample weight	Radioactivity	Sample weight	Radioactivity
mg.	*c.p.m.*	*mg.*	*c.p.m.*	*mg.*	*c.p.m.*	*mg.*	*c.p.m.*
6.1	3100	6.1 (92%)§	3050 (97%)	7.9 (100%)	3050 (97%)	4.7 (96%)	none¶
5.3	2820	5.6 (96%)	2510 (96%)	6.3 (92%)	2570 (94%)	4.1 (96%)	none¶
6.2	3100	6.2 (91%)	3030 (95%)	7.5 (94%)	3030 (95%)	4.8 (96%)	none¶

* Determined spectrophotometrically.

† Determined gravimetrically.

‡ Calculated from a standard curve using *L. arabinosus* as the test organism.

§ Percentage yields calculated on a molar basis.

¶ Less than 15 c.p.m.

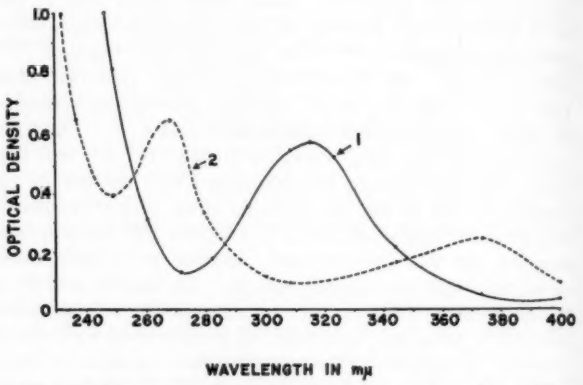

FIG. 5. Absorption curves for HAA (*Curve 1*) and metaphosphoric acid filtrate (*Curve 2*) from a 45-minute incubation period.

in the enzyme system inactivated by acid showed a complete disappearance of substrate (Table II) in all three experiments carried out with labeled HAA. This was in agreement with the observed shift in the spectral curve from 315 mμ, the peak of maximal absorption for HAA, to 268 mμ, the peak of maximal absorption of quinolinic acid (Fig. 5). The slight peak at 372 mμ was due to the presence of a very small amount of unreacted intermediate (8). Essentially all of the radioactivity was found in the quinolinic acid formed (Table II).

The spectral analysis of the eluate from the Dowex 1 anion exchange column was identical to that of pure quinolinic acid, and the amount of radioactivity found indicated that the labeled carbon 3 of the HAA was undiluted in the quinolinic acid formed.

The quinolinic acid was decarboxylated with glacial acetic acid at 125° for 35 minutes and the evolved CO_2 was trapped in a saturated $Ba(OH)_2$ solution as $BaCO_3$. Purified, CO_2-free nitrogen was used to flush all the CO_2 formed into the trap and under these conditions, the evolved CO_2 was obtained quantitatively. The $BaCO_3$-$Ba(OH)_2$ solution was immediately filtered onto a 25-mm. filter paper disc, with the use of a Tracerlab E-8B stainless steel precipitation apparatus, washed with a small

amount of cold water, and finally washed with acetone. The dried disc containing the $BaCO_3$ was weighed and counted for radioactivity. The results are summarized in Table II. The amount of CO_2 formed was consistent with the theoretical amount of quinolinic acid formed from the HAA and the results indicate that all of the radioactivity was present in the α-carboxyl group of the quinolinic acid.

No nicotinic acid was detected in the test samples before decarboxylation of the quinolinic acid. The nicotinic acid solution obtained from the decarboxylation of the quinolinic acid was purified on a Dowex 50 cation exchange column, with 0.75 N HCl as the eluting agent. No radioactivity was detected in the nicotinic acid fraction and the amount of nicotinic acid produced was equal to the determined amount of quinolinic acid initially formed by the enzyme system.

DISCUSSION

If the mechanism of conversion of HAA to quinolinic acid does occur through cleavage between carbon atoms 3 and 4 of its benzene ring, carbon 3 should form the α-carbon atom of the quinolinic acid. This carbon atom would then be evolved as CO_2 from the decarboxylation of quinolinic acid to nicotinic acid. The amount of radioactivity found in the quinolinic acid and in the CO_2 after decarboxylation of quinolinic acid clearly demonstrates that the CO_2 is derived from carbon 3 of HAA in the enzymatic conversion of HAA to quinolinic acid. No radioactivity was found in the nicotinic acid and since the amount of nicotinic acid formed was in agreement with the theoretical quantity that should have been produced, picolinic acid, if present was a minor product. The amount of quinolinic acid formed was also in agreement with the amount of substrate metabolized, as measured photofluorometrically and spectrophotometrically. The postulated scheme of the conversion of HAA to nicotinic acid is shown in Fig. 6.

Wiss and Bettendorf (9) have demonstrated that the HAA takes up oxygen to form the postulated intermediate, compound XI. This may be expected to tautomerize to an acroleinmaleic acid derivative, compound XII, and then to form quinolinic acid, compound XIII, spontaneously or enzymatically or both. The quinolinic acid is then decarboxylated to form nicotinic acid, compound XIV, and CO_2.

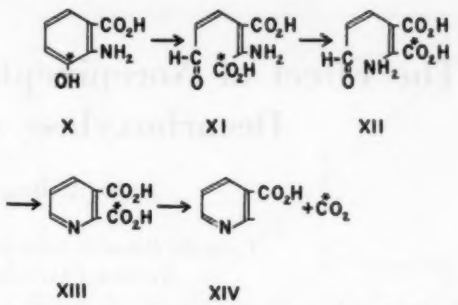

FIG. 6. Postulated scheme of the conversion of HAA to nicotinic acid.

SUMMARY

1. A chemical synthesis of 3-hydroxyanthranilic acid-3-C^{14} from phenol-1-C^{14} was devised.

2. A method for the spectrophotometric determination of quinolinic acid has been developed after purification of quinolinic acid by chromatography.

3. 3-Hydroxyanthranilic acid-3-C^{14}, incubated at 37.5° with an enzyme preparation from rat liver, was quantitatively converted to quinolinic acid, which, after purificatoin was shown to contain all of the radioactivity in its α-carboxyl group without dilution.

4. Quinolinic acid-α-carboxy-C^{14} was quantitatively decarboxylated nonenzymatically to form $C^{14}O_2$ and unlabeled nicotinic acid.

5. A mechanism for the conversion of 3-hydroxyanthranilic acid to quinolinic acid has been postulated tracing the change of carbon atom 3 of 3-hydroxyanthranilate to the α-carboxyl group of quinolinic acid and then to CO_2. All of the original radioactivity was shown to be in the CO_2 collected.

REFERENCES

1. BONNER, D., *Proc. Natl. Acad. Sci. U. S.*, **34**, 5 (1948).
2. MITCHELL, H. K., AND NYC, J. F., *Proc. Natl. Acad. Sci.*, **34**, 1 (1948).
3. HENDERSON, L. M., AND HANKES, L. V., *J. Biol. Chem.*, **222**, 1069 (1956).
4. HEIDELBERGER, C., ABRAHAM, E. P., AND LEPKOVSKY, S., *J. Biol. Chem.*, **179**, 151 (1949).
5. SCHAYER, R. W., AND HENDERSON, L. M., *J. Biol. Chem.*, **195**, 657 (1952).
6. SCHWEIGERT, B. S., *J. Biol. Chem.*, **178**, 707 (1949).
7. BOKMAN, A. H., AND SCHWEIGERT, B. S., *J. Biol. Chem.*, **180**, 153 (1950).
8. MIYAKE, A., BOKMAN, A. H., AND SCHWEIGERT, B. S., *J. Biol. Chem.*, **211**, 391 (1954).
9. WISS, O., AND BETTENDORF, G., *Z. physiol. Chem.*, **306**, 145 (1957).
10. MEHLER, A. H., *J. Biol. Chem.*, **218**, 241 (1956).
11. MAKINO, K., ITOH, F., AND NISHI, K., *Nature*, **167**, 115 (1951).
12. CASON, J., AND RAPOPORT, H., *Laboratory text in organic chemistry*, Prentice-Hall, Inc., New York, 1950.
13. SANNIE, M. C., *Bull. soc. chim. France*, **17**, 322 (1950).
14. INGOLD, C. K., AND INGOLD, E. H., *J. Chem. Soc.*, 1310, 1926.
15. PHILLIPS, M. A., *J. Chem. Soc.*, 2685, 1930.
16. SANNIE, M. C., AND LAPIN, H., *Bull. soc. chim. France*, **19**, 369 (1952).

The Effect of Norepinephrine on the 5-Hydroxytryptophan Decarboxylase Activity of Rat Kidney*

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The implication of 5-hydroxytryptamine in a wide variety of metabolic and physiologic functions (1) suggested a study of factors influencing the biosynthesis of this amine. The rate of biosynthesis of 5-hydroxytryptamine can be accelerated *in vitro* or *in vivo* by addition of the precursor, 5-hydroxytryptophan (2), or the appropriate form of the coenzyme, pyridoxal phosphate (3, 4). Inhibition of 5-hydroxytryptophan decarboxylase by carbonyl reagents (3, 5, 6), metal-chelating agents (6), cupric ions (3), phenylalanine derivatives (7), and substituted indoles (8) has been reported. Nonspecific inhibition by sulfhydryl-characterizing reagents has also been demonstrated (3).

In the course of a study of the effects of various compounds on the 5-hydroxytryptophan decarboxylase activity of rat kidney homogenates, norepinephrine was found to be inhibitory. In view of certain interesting postulates regarding the role of norepinephrine and of 5-hydroxytryptamine (9), the mechanism of the observed inhibition was investigated in detail. Kinetic and spectral analysis revealed that the inhibition of the decarboxylase by norepinephrine is formally noncompetitive, involving the removal of the coenzyme, pyridoxal phosphate, through nonenzymatic combination with the inhibitor. The product of this interaction has been prepared and analyzed.

EXPERIMENTAL

Methods—The 5-hydroxytryptophan decarboxylase activity of rat kidney homogenates was determined by measuring the 5-hydroxytryptamine formed during a 2-hour anaerobic incubation at pH 8.0 in 0.1 M sodium pyrophosphate buffer. The incubation mixture consisted of 270 μmoles of buffer, 10 μmoles of 5-hydroxytryptophan, 0.1 μmole of pyridoxal phosphate, and 20 to 25 mg. dry weight of the homogenate in a final volume of 3.0 ml. Details of the incubation and the analysis have been reported elsewhere (3). The compounds studied were added to the incubation mixture to give the final concentrations indicated. All activity data are in terms of μmoles of 5-hydroxytryptamine formed per 100 mg. of dry weight per hour.

Examinations of the ultraviolet spectra were carried out in 1-cm. silica cells with a Beckman model DU spectrophotometer. Infrared analyses were obtained with a Perkin-Elmer model 21 spectrophotometer.

Materials—The compounds used were obtained from the following sources: amphetamine (Smith, Kline and French Laboratories), norepinephrine (Mann Research Laboratories, Inc. and Winthrop Laboratories), epinephrine, tyramine hydrochloride, 5-hydroxytryptamine creatinine sulfate, and 5-hydroxy-DL-tryptophan (Nutritional Biochemicals Corporation), 3-hydroxytyramine (California Foundation for Biochemical Research), phenylpropanolamine hydrochloride (Merck, Sharp and Dohme), *m*-hydroxyphenylpropanolamine (Dr. M. L. Tainter, Sterling-Winthrop Research Institute), and phenylethylamine (Eastman Organic Chemicals).

RESULTS

During an investigation of the effect of a wide variety of compounds on the 5-hydroxytryptophan decarboxylase activity of rat kidney homogenates significant inhibition was obtained with norepinephrine. Further study showed that this inhibition occurred only in the presence of added codecarboxylase (Fig. 1). In an attempt to characterize the inhibition, the absorption spectra of incubation mixtures in which the reaction was stopped by heating were examined. It was found that the pyridoxal phosphate absorption maximum near 390 mμ had disappeared during the incubation and had been replaced by a new maximum at 320 mμ. Similar results were obtained when norepinephrine and codecarboxylase were incubated together in the absence of the homogenate under the conditions of the enzymatic studies (Fig. 2).

These results suggested a direct reaction between codecarboxylase and norepinephrine and recalled the inhibition of 3,4-dihydroxyphenylalanine decarboxylase by an excess of the substrate or related compounds (10–12) and the inhibition of cysteinesulfinic and cysteic acid decarboxylases by cysteine (13). These latter inhibitions have been ascribed to an interaction of the inhibitor with codecarboxylase and spectrophotometric evidence of such reactions has been presented (10, 13). One type of reaction between norepinephrine and pyridoxal was studied by Heyl *et al.* (14), who observed that these compounds interacted under reducing conditions to form an *N*-pyridoxyl derivative. This group inferred that if reduction of the intermediate Schiff base were not carried out immediately, rearrangement to a tetrahydroisoquinoline would have occurred. Schott and Clark (10) prepared and characterized a tetrahydroisoquinoline from *m*-hydroxyphenylpropanolamine and semipurified codecarboxylase and, by comparing its spectral characteristics with those of reaction mixtures containing the cofactor and other phenylethylamines which included norepinephrine, assumed that the norepinephrine-codecarboxylase product was tetrahydroisoquinoline. These workers, however, were unable to isolate the product of any of the latter reactions.

* A brief report of this work was presented before the American Society of Biological Chemists, April 13–18, 1958, in Philadelphia, Pennsylvania.

A second possibility was suggested by the similarity between the spectral changes observed in the present study and those reported as occurring during the inhibition of cysteinesulfinic and cysteic acid decarboxylases by cysteine (13). In this latter case the inhibition was ascribed to nonenzymatic formation of a thiazolidine from cysteine and pyridoxal phosphate. The possible formation of an analogous oxazolidine derived from norepinephrine and pyridoxal phosphate could not be neglected in the present studies in view of the report of oxazolidine formation from L-ephedrine and benzaldehyde (15).

Finally, it was thought that a Schiff base might have been the product of the observed reaction between pyridoxal phosphate and norepinephrine. To choose among these possibilities the following studies were undertaken.

Mechanism of Reaction—The reactive groups involved in the observed reaction were indicated by the following. When epinephrine and pyridoxal phosphate were incubated under the conditions described no alterations of the spectral characteristics were noted. Although pyridoxal phosphate gives a positive carbonyl reaction with phenylhydrazine, this carbonyl test was negative with the incubated mixture of norepinephrine and codecarboxylase. These findings suggested a reaction between the amino group of norepinephrine and the carbonyl group of codecarboxylase. The reaction was found to occur readily at pH 7.4 to 8.0 in barbital, phosphate, or pyrophosphate buffers in the presence or absence of air.

The three possible reactions cited above, all of which involve a free amino group and the loss of a carbonyl, were then considered. First, a Schiff base as the product of the reaction between codecarboxylase and the amino group was rendered unlikely by the recent report of Matsuo (16) that such reactions, under conditions similar to those used here, cause a shift of the 390 mμ peak to higher wave lengths rather than toward 320 mμ. Next, the formation of an oxazolidine was ruled out on the basis of the results obtained with a series of phenethylamines which indicated that the secondary alcohol moiety of norepinephrine is not essential for reaction of the phenethylamine with codecarboxylase (Table I).

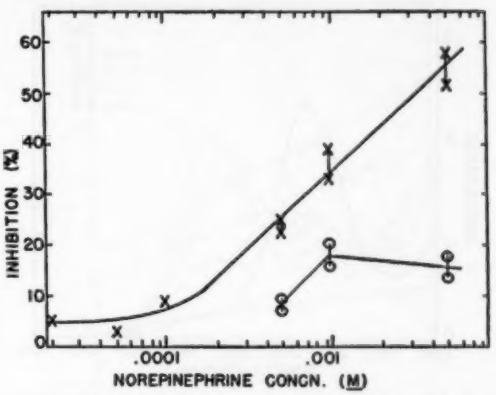


FIG. 1. The effect of norepinephrine on 5-hydroxytryptophan decarboxylase in the presence, ×——×, or absence, O——O, of added codecarboxylase (0.1 μmole per 3 ml.)

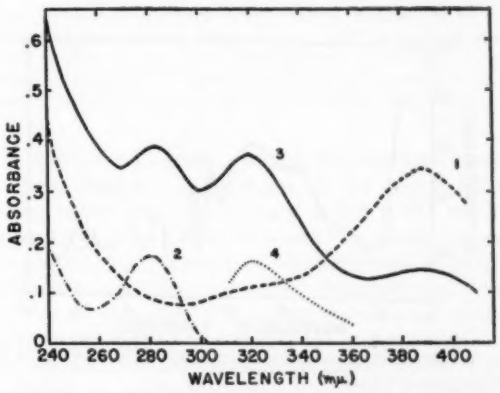
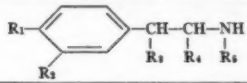


FIG. 2. Ultraviolet absorption spectra of 0.2 μmole each of pyridoxal phosphate (*Curve 1*) and norepinephrine (*Curve 2*), the nonenzymatic reaction mixture (*Curve 3*), and the heat-inactivated incubation mixture (*Curve 4*).

TABLE I

Reactivity of substituted phenethylamines with codecarboxylase and inhibition of 5-hydroxytryptophan decarboxylase

Conditions: Reactivity with codecarboxylase was estimated by the decrease in absorbance at 390 mμ when 0.2 μmole each of pyridoxal phosphate and the test compound were incubated together for 2 hours in N_2 at room temperature in 0.1 M sodium pyrophosphate buffer, pH 8.0. Enzymatic inhibition was determined as outlined under "Methods." Activity of control homogenates was in the range 3.24 to 4.66 μmoles of 5-hydroxytryptamine per 100 mg. per hour.

Compound						Reactivity with codecarboxylase	5-Hydroxytryptophan decarboxylase inhibition	
	R_1	R_2	R_3	R_4	R_5	−Δ390 mμ Absorbance	1×10^{-3} M	5×10^{-3} M
							%	%
Phenylethylamine	H	H	H	H	H	0.013	None	
Tyramine	OH	H	H	H	H	0.035	None	
m-Hydroxytyramine	OH	OH	H	H	H	0.224	39	53
Norepinephrine	OH	OH	OH	H	H	0.275	33	52
Epinephrine	OH	OH	OH	H	CH_3	0.017	None	None
Phenylpropanolamine	H	H	OH	CH_3	H	0.000	None	
m-Hydroxyphenylpropanolamine	H	OH	OH	CH_3	H	0.254	29	63
3,4-Dihydroxyphenylalanine	OH	OH	H	COOH	H	0.216	57	78
Amphetamine	H	H	H	CH_3	H	0.050	None	

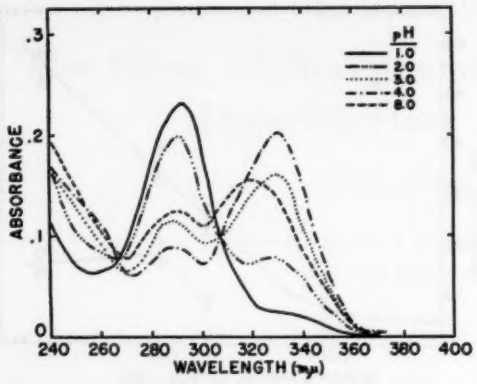


FIG. 3. Ultraviolet absorption spectra of the synthetic tetrahydroisoquinoline. Solvents used were HCl, pH 1.0, phosphate buffer, pH 2.0, citrate buffer, pH 3.0, formate buffer, pH 4.0, and pyrophosphate buffer, pH 8.0. In all cases, 100 mg. of the compound per l. of 0.1 M solvent.

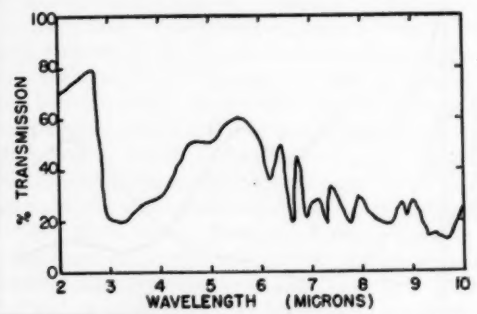


FIG. 4. Infrared absorption spectrum of the synthetic tetrahydroisoquinoline in KBr.

The structural characteristics required in the substituted phenethylamine for reaction with pyridoxal phosphate under the described conditions are (*a*) the presence of a phenolic group *meta* to the aliphatic side chain, and (*b*) a free primary amino group in the terminal position of the 2-carbon side chain (Table I). These characteristics are typical of those involved in a Pictet-Spengler isoquinoline synthesis (17) and suggest that the product of the norepinephrine-codecarboxylase reaction is indeed a substituted tetrahydroisoquinoline. This is in agreement with the inferences of Schott and Clark (10) and Heyl *et al.* (14).

In order to obtain a more rigorous proof of the identity of the presumed tetrahydroisoquinoline, an authentic sample of the compound was prepared from pyridoxal phosphate and norepinephrine.

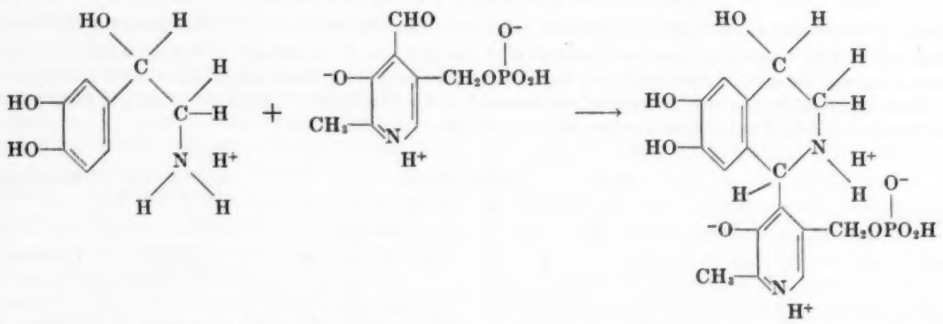
Synthesis of 2-Methyl-3-hydroxy-4-(4,6,7-trihydroxy-1,2,3,4 tetrahydroisoquinolyl)-5-pyridylmethyl Phosphate—A solution of pyridoxal phosphate, 1.50 gm. (5.66 mmoles) in 18 ml. of 4 per cent sodium bicarbonate, was added to a solution of 1.16 gm. (5.64 mmoles) of norepinephrine hydrochloride in 4 ml. of water and agitated with nitrogen gas at room temperature for 1 hour. The reaction mixture was chilled, the heavy yellow precipitate collected on a filter, and washed successively with iced water, ethanol, and diethyl ether. The crude material was recrystallized once from hot water with Norit. A pale, lemon yellow, hygroscopic product was obtained in 39 per cent over-all yield (0.95 gm.) which darkened above 180° and decomposed in the range 210 to 215°. Analytical figures are as follows (given as per cent):

$$C_{16}H_{19}N_2O_8P.3H_2O\ (452.367)$$

Calculated:	C 42.48, H 5.57, N 6.19, P 6.85
Found:	C 42.30, H 5.27, N 6.34, P 6.72

The product had a primary ultraviolet absorption maximum at 320 mμ in 0.1 M sodium pyrophosphate buffer, pH 8.0 (Fig. 3). An infrared analysis of the product in KBr showed broad absorption bands in the regions 2000 to 3700 cm.$^{-1}$ and 1000 to 1110 cm.$^{-1}$ with individual absorption peaks at 1620, 1455, 1375, 1285, 772, and 742 cm.$^{-1}$ (Fig. 4).

The method of preparation conforms essentially to one set of conditions for a Pictet-Spengler isoquinoline synthesis (17). Other products possessing the same elemental composition that might have been produced under these conditions, *i.e.* an oxazolidine or a Schiff base, were improbable in view of the effect of pH on the absorbance characteristics of the product (Fig. 3). With increasing acidity, the major absorption maximum shifted from 320 mμ to 290 mμ, a change typical of pyridoxine derivatives (18, 19). The original maximum at 320 mμ was restored upon neutralization to pH 4 to 8. Stability to acidification is not characteristic of oxazolidines (15) or Schiff bases (20). The reaction indicated is:



Comparison of the spectral characteristics of the product obtained in solution during the enzyme inhibition studies (Fig. 2) and those of the authentic tetrahydroisoquinoline (Fig. 3) indicated the identity of these two compounds. Concentrations of the synthetic tetrahydroisoquinoline as high as 10^{-2} M were found to be without effect on the 5-hydroxytryptophan decarboxylase activity of rat kidney homogenates; this demonstrated that the observed inhibition by norepinephrine is to be attributed to re-

moval of codecarboxylase and not to a direct effect of the tetrahydroisoquinoline.

Good qualitative correlation could be demonstrated with respect to the ability of various phenethylamines to react nonenzymatically with pyridoxal phosphate and their ability to inhibit the decarboxylase, although quantitative agreement was less apparent (Table I). The best inhibitor, 3,4-dihydroxyphenylalanine, is not the most reactive with codecarboxylase. This might, however, be related to the fact that dihydroxyphenylalanine is also decarboxylated by these crude kidney preparations. The activity of dihydroxyphenylalanine decarboxylase would increase the requirement for added codecarboxylase and lead to the production of *m*-hydroxytyramine, another potent inhibitor. The apparent lack of quantitative correlation could also be related to variation in the rates of metabolic destruction of the several inhibitors by the kidney homogenates.

Mechanism of Enzymatic Inhibition by Norepinephrine—When codecarboxylase was mixed with an excess of the substrate 5-hydroxytryptophan under the conditions of the enzymatic reaction, an immediate shift of the pyridoxal phosphate absorption maximum from 390 mμ to 410 mμ was noted. This spectral change is similar to that reported for the formation of Schiff bases between codecarboxylase and other amino acids under similar conditions (16). After it had been established that codecarboxylase reacts with both inhibitor and substrate, its apparent relative affinity for these compounds was determined.

Aerobic incubation of 0.2 μmole each of norepinephrine, 5-hydroxytryptophan, and codecarboxylase for 2 hours at room temperature in pH 8.0 sodium pyrophosphate buffer (0.1 M) resulted in the loss of codecarboxylase (390 mμ) and the appearance of the tetrahydroisoquinoline (320 mμ). Preincubation of hydroxytryptophan and codecarboxylase for 30 minutes before the addition of norepinephrine, or the use of 100-fold excess of hydroxytryptophan failed to reverse or prevent the formation of the norepinephrine-codecarboxylase product. These results indicated that the inhibitor-cofactor product is decidedly more stable than the substrate-cofactor product and offered a satisfactory explanation for the enzymatic inhibition observed. On the basis of these nonenzymatic studies, the inhibition of the decarboxylase by norepinephrine was considered to be of a noncompetitive nature.

The effect of substrate concentration on the enzymatic inhibition by norepinephrine was determined. A suitable analysis (21) of these data demonstrated that norepinephrine is formally a noncompetitive inhibitor of 5-hydroxytryptophan decarboxylase. By graphic estimation, $K_m = 2.5 \times 10^{-5}$ M and the *apparent* $K_i = 5 \times 10^{-3}$ M (Fig. 5).

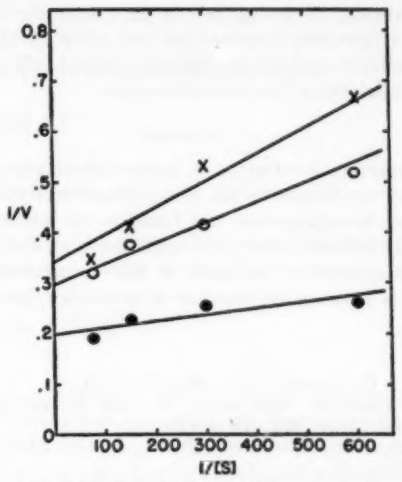


FIG. 5. Reciprocal curve analysis showing noncompetitive inhibition of 5-hydroxytryptophan decarboxylase activity by norepinephrine. Control, ●——●; 1×10^{-3} M norepinephrine, ○——○; and 5×10^{-3} M norepinephrine, ×——×. Velocity expressed as μmoles of 5-hydroxytryptamine formed per 100 mg. per hour and substrate concentration as moles per liter.

DISCUSSION

The results reported here provide additional evidence of the essentiality of pyridoxal phosphate for 5-hydroxytryptophan decarboxylase. They also suggest an explanation for the observation that although activation by added pyridoxal phosphate of this enzyme, obtained from rat kidney, occurred readily in phosphate or pyrophosphate buffers, it could not be demonstrated in Tris[1] or glycylglycine buffers.[2] In these latter instances the added codecarboxylase was apparently inactivated by Schiff base formation with the buffer which was present in large excess as compared with the substrate. Indeed, the absorption maximum of pyridoxal phosphate at 390 mμ in pH 8.0 pyrophosphate or phosphate buffers was found to have shifted to 415 to 420 mμ if Tris or glycylglycine buffers were used.[2] A similar finding has been encountered by Matsuo (16), who demonstrated Schiff base formation between Tris and pyridoxal phosphate and cited the lack of normal activation of homoserine deaminase by the cofactor when incubations were carried out in Tris buffer.

The demonstration that the addition of 5-hydroxytryptophan to a solution of pyridoxal phosphate produces a spectral shift similar to those produced in the formation of Schiff bases from pyridoxal phosphate and other amino acids (16) suggests that the decarboxylase functions through a pyridoxal phosphate:hydroxytryptophan Schiff base intermediate in a fashion similar to that proposed for other decarboxylases (22). The failure of norepinephrine to be equally effective as an inhibitor in the presence or absence of added codecarboxylase (Fig. 1) suggests that the aldehyde group of the endogenous cofactor may be protected from the reaction with the amine by combination with the apoenzyme. Steric effects or specificity of binding sites might prevent the reaction of norepinephrine with bound codecarboxylase.

Although, in a nonenzymatic system, codecarboxylase and the various inhibitors react readily when combined in a 1:1 ratio, effective enzymatic inhibition is obtained only when a 30-fold excess of inhibitor is present. Enzymatic destruction of the inhibitors would be expected to reduce their concentration and rapid combination of the added cofactor with the apoenzyme would also limit the exposure of free codecarboxylase to the inhibitor.

Although the concentrations of norepinephrine required to produce significant inhibition of the enzymatic activity *in vitro* are quite high, the demonstration that the catechol amine reacts readily with free codecarboxylase when equimolar amounts of the two are mixed under seemingly physiological conditions, permits speculation on the possibility that norepinephrine and

[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

[2] J. A. Buzard and P. D. Nytch, unpublished observations.

related naturally occurring amines may alter the activity of 5-hydroxytryptophan decarboxylase and other pyridoxal phosphate-dependent enzymes in certain specialized cells by regulating the availability of free codecarboxylase.

SUMMARY

In a continuing investigation of factors affecting the formation of 5-hydroxytryptamine by rat kidney 5-hydroxytryptophan decarboxylase, norepinephrine was found to be inhibitory. Detailed study indicated that norepinephrine is a nonspecific, formally noncompetitive inhibitor of the decarboxylase. This inhibition is achieved by removal of codecarboxylase added to the system *in vitro* through nonenzymatic reaction with norepinephrine. This conclusion was strengthened by the preparation and characterization of the product of the reaction between pyridoxal phosphate and norepinephrine and its identification as a substituted tetrahydroisoquinoline. The possible significance *in vivo* of this inhibition is discussed.

Acknowledgment—Elemental analyses on the synthetic product were kindly performed by Mr. G. Ginther. The authors are also indebted to Dr. J. Howard for the infrared absorption data reported.

REFERENCES

1 Page, I. H., *Physiol. Revs.*, **38**, 277 (1958).
2. Udenfriend, S., Weissbach, H., and Bogdanski, D. F., *J. Biol. Chem.*, **224**, 803 (1957).
3. Buzard, J. A., and Nytch, P. D., *J. Biol. Chem.*, **227**, 225 (1957).
4. Buzard, J. A., and Nytch, P. D., *J. Biol. Chem.*, **229**, 409 (1957).
5. Clark, C. T., Weissbach, H., and Udenfriend, S., *J. Biol. Chem.*, **210**, 139 (1954).
6. Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, **211**, 39 (1954).
7. Davison, A. N., and Sandler, M., *Nature*, **181**, 186 (1958).
8. Freter, K., Weissbach, H., Redfield, B., Udenfriend, S., and Witkop, B., *J. Am. Chem. Soc.*, **80**, 983 (1958).
9. Brodie, B. B., in H. A. Abramson (Editor), *Neuropharmacology, Vol. III*, Josiah Macy, Jr., Foundation, New York, 1957, p. 323.
10. Schott, H. F., and Clark, W. G., *J. Biol. Chem.*, **196**, 449 (1952).
11. Sourkes, T. L., *Rev. can. biol.*, **14**, 49 (1955).
12. Gonnard, P., *Bull. soc. chim. biol.*, **32**, 535 (1950).
13. Bergeret, B., Chatagner, F., and Fromageot, C., *Biochim. et Biophys. Acta*, **22**, 329 (1956).
14. Heyl, D., Luz, E., Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, **74**, 414 (1952).
15. Bergmann, E. D., *Chem. Revs.*, **53**, 309 (1953).
16. Matsuo, Y., *J. Am. Chem. Soc.*, **79**, 2011 (1957).
17. Whaley, W. M., and Govindachari, T. R., *Org. Reactions*, **6**, 151 (1951).
18. Harris, S. A., Webb, T. J., and Folkers, K., *J. Am. Chem. Soc.*, **62**, 3198 (1940).
19. Williams, V. R., and Neilands, J. B., *Arch. Biochem. Biophys.*, **53**, 56 (1954).
20. Allen, C. F. H., and Blatt, A. H., in H. Gilman (Editor), *Organic chemistry, an advanced treatise, Vol. I, 2nd. Edition*, John Wiley and Sons, Inc., New York, 1943, p. 659.
21. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
22. Metzler, D. E., Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, **76**, 648 (1954).

Reaction of Ethylene Oxide with Nicotinamide and Nicotinic Acid*

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It has been shown that treatment of animal diets with ethylene oxide results in severe destruction of thiamine, niacin, pyridoxine, riboflavin, and folic acid (2, 3) and of the essential amino acids, histidine, methionine (4), and lysine (5). Consequently, studies were undertaken to elucidate the nature of the reactions of ethylene oxide with the above nutrients. The realization that a tertiary nitrogen group was common to six of the labile nutrients prompted the investigation of the reaction of ethylene oxide with pyridine and the closely related nicotinamide and nicotinic acid.

Lohmann (6), in 1939, reported that the addition of ethylene oxide to pyridine or quinoline results in the formation of highly colored methanol-water soluble products of undetermined structure. Eastham *et al.* (7–9) demonstrated that ethylene oxide reacts as readily with tertiary as with primary and secondary amines, and that the reaction with pyridine, in the presence of an acid, yields the N^1-(2-hydroxyethyl)pyridinium salt. The rate of reaction in excess pyridine was shown to be second order, being proportional to the concentration of acid and epoxide.

The present paper offers evidence that ethylene oxide reacts with nicotinamide under very mild conditions to yield, after acidification with HCl, the chloride of N^1-(2-hydroxyethyl)-nicotinamide, and with nicotinic acid to produce the betaine of N^1-(2-hydroxyethyl)nicotinic acid.

EXPERIMENTAL

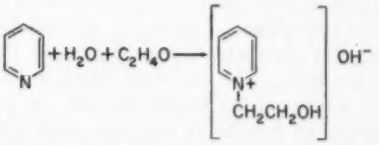
Reaction Conditions—Unless otherwise indicated, the reactions with ethylene oxide (b.p. 11°) were carried out as follows. A beaker containing an aqueous solution of the compound to be treated was placed in a 4-l. desiccator, which then was evacuated to 40 mm. of Hg. The pressure in the desiccator was restored to ambient by admitting ethylene oxide from a tank of the compressed gas.[1] As ethylene oxide dissolved in the solution, additional gas was admitted to maintain ambient pressure. After about 20 minutes of exposure, the liquid and gaseous phases reached equilibrium and subsequent gas uptake was very slow. Projecting into the desiccator through a rubber stopper in the top were the glass electrodes of a pH meter and the extended tip of a burette, thereby enabling adjustment of pH to be made when desired. Reactions were conducted at room temperature.

Determination of Nicotinic Acid and N^1-Methylnicotinamide Activity—Nicotinic acid activity was determined by the method of Dann and Satterfield (10) with the test organism *Lactobacillus arabinosus* 17–5, ATCC 8014. The fluorometric determination of N^1-methylnicotinamide activity was carried out by the method of Huff and Perlzweig (11).

RESULTS

Reaction of Ethylene Oxide with Pyridine—When pyridine (110 mmoles in a 1:1 aqueous solution, pH 9.5) was treated with ethylene oxide, the clear solution became light brown after 1 hour, and at the end of 2 hours it was almost black and quite viscous. Reaction in the vapor phase had deposited some of the dark, oily product on the walls of the desiccator. The pH was greater than 12; 80 mmoles of HCl were required to return the pH to 9.5. Upon acidification, the pyridine product became deep yellow, the color change being freely reversible by addition of alkali. A deep red, highly viscous syrup was deposited when the solvent was removed from the acidified reaction mixture under vacuum. It was highly soluble in water and 95 per cent ethanol, but insoluble in ethyl ether and acetone.

When pyridine (reagent grade) was treated with ethylene oxide without the addition of water, similar products were obtained, but the rate of reaction was much slower; no noticeable discoloration of the pyridine was seen until after 4 hours. This is consistent with the observation of Knorr (12), who reported the failure of ethylene oxide to react with amines in the absence of water or ethanol as solvents. According to Eastham's formulation (8), water serves as a proton donor in the reaction with pyridine:



The high alkalinity produced suggested the formation of a quaternary pyridinium hydroxide. The instability of these compounds has been recognized (13), and no further attempt was made to purify or characterize the products of the reaction.

* A preliminary report of this work was presented before the annual meeting of the American Society of Biological Chemists (1). The work done at Virginia Polytechnic Institute was supported by Grant No. A 1099 from the National Institutes of Health.

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[1] Gaseous ethylene oxide (99.15 per cent purity) was purchased from Matheson Company, Inc., East Rutherford, New Jersey.

TABLE I

Paper chromatography of products isolated from ethylene oxide-nicotinamide reaction

Solvent	Spotted at origin	R_F as detected by	
		Bromophenol blue	Fluorescence under ultraviolet
1-Butanol saturated with H_2O	Reaction products	0.58	0.00
	Triethanolamine	0.58	None
	Reaction products + triethanolamine	0.58	0.00
1-Propanol:H_2O (7:3)	Reaction products	0.74	0.15 (streak)
	Triethanolamine	0.73	None
	Reaction products + triethanolamine	0.73	0.15 (streak)

Reaction of Ethylene Oxide with Nicotinamide—When an aqueous solution of nicotinamide was first treated with ethylene oxide, no effort was made to curb the rising pH of the reaction mixture. As a result, the product isolated was not the anticipated N^1-(2-hydroxyethyl)nicotinamide, which we now know to be unstable in alkali, but rather the product of a secondary reaction. The following details apply to the reaction in which the pH was uncontrolled.

The reaction of ethylene oxide with 40.9 mmoles of nicotinamide in 30 ml. of distilled water (initial pH, 7.4) for 20 hours resulted in a deep red, strongly alkaline solution (> pH 12) which fluoresced under ultraviolet irradiation and evolved ammonia. The pH was readjusted to 7.4 with 29.6 mmoles of HCl, and the solution was concentrated under vacuum to a deep red, highly viscous residue which was taken up in a minimal quantity of hot 50 per cent ethanol. Acetone was added to the point of incipient cloudiness. A mass of fine red needles precipitated from this solution after it was cooled for a few hours at 5°. The product was recrystallized twice from 50 per cent ethanol and acetone yielding 1.4 gm. of rust-colored needles, m.p. 176–178°.

The melting point (14) and analysis[2] coincide closely with those of triethanolamine hydrochloride.

$C_6H_{14}NO_3Cl$

Calculated: C 38.80, H 8.70, N 7.55, Cl 19.10

Found: C 39.22, H 8.07, N 7.60, Cl 19.19

It was suspected that the product was contaminated with a trace of a red-colored impurity. This hypothesis was supported by the observation that paper chromatography resolved the product into two spots, a yellow one which fluoresced under ultraviolet irradiation and a basic spot which could be detected by spraying the developed paper with slightly acidic bromophenol blue in 95 per cent ethanol. Authentic triethanolamine (Eastman Kodak) was chromatographed singly and together with the reaction products. The results are shown in Table I.

Two likely sources of the triethanolamine can be suggested: (*a*) The reaction of 3 moles of ethylene oxide with ammonia released from the amide group of the nicotinamide at the elevated pH. (*b*) The reaction of 2 moles of ethylene oxide with ethanolamine, which was identified later as an alkaline degradation product of the initially formed N^1-(2-hydroxyethyl) nicotinamide.

To eliminate the accumulation of an unstable quaternary pyridinium hydroxide, the pH of the reaction mixture in the next experiment was controlled by the continuous addition of HCl.

pH-Controlled Reaction of Ethylene Oxide with Nicotinamide—A 30-ml. aqueous solution of 40.9 mmoles of nicotinamide was treated with ethylene oxide, and the pH was maintained between 7 and 10.5 by the dropwise addition of 2 N HCl. Initially, the rise in pH was rapid, and addition of acid was almost continuous. After 6 hours of reaction at controlled pH, further increase in alkalinity was slow and the reaction was terminated. The pale yellow solution was decolorized with charcoal and Attaclay,[3] and concentrated to a white crystalline mass, which was recrystallized twice from aqueous ethanol and acetone, yielding 3.3 gm. (40 per cent) of white, glistening platelets, m.p. 195.5–196°.

$C_8H_{11}O_2N_2Cl$

Calculated: C 47.40, H 5.44, N 13.81, Cl 17.50

Found: C 47.76, H 5.76, N 14.01, Cl 17.48

A colorless aqueous solution of this compound made strongly alkaline with NaOH becomes deep red, fluoresces under ultraviolet irradiation, and evolves ammonia. The compound is highly soluble in water and slightly soluble in ethanol (about 2 mg. per ml. at 5°).

Support for Structure of Ethylene Oxide Nicotinamide-HCl Product—All evidence suggests that this product is the chloride of N^1-(2-hydroxyethyl)nicotinamide. The compound appears pure when chromatographed on paper and can be detected by its strong quenching of ultraviolet irradiation. Spots on paper fluoresce strongly under ultraviolet irradiation after being sprayed with 1 M aqueous KCN (15, 16), or when exposed to vapors of ethyl-methyl ketone and NH_3 (1:1), the latter reaction being typical of quaternary pyridinium compounds with a side chain CO—NHR in the β position (17). The compound fluoresces when its solutions are assayed for N^1-methylnicotinamide by the fluorometric method of Huff and Perlzweig (11), its molar fluorescence being 2.08 times that of N^1-methylnicotinamide. This fluorometric assay was used in subsequent experiments for quantitatively determining the product.

Ethanolamine is a product of the alkaline degradation of this compound. Refluxing 1 mmole in 25 ml. of 25 per cent aqueous KOH for 14 hours produced a deep orange solution. Vacuum distillation yielded a clear distillate which contained 0.55 mmole of a primary amine, as determined by the manometric method of Van Slyke (18). Another assay involved the oxidation of the amine to formaldehyde with slightly alkaline periodate, and the resulting formaldehyde was quantitatively determined by the colorimetric chromotropic acid procedure of Critchfield and Johnson (19). A yield of 0.52 mmole was obtained, the method being standardized with authentic ethanolamine. Oxidation by periodate implies that the amine is a 1,2-hydroxyamine or a related compound (20). The amine had R_F values on paper identical with those of authentic ethanolamine in four solvent systems: (*a*) H_2O-saturated 1-butanol, R_F 0.11; (*b*) 1-butanol saturated with 3 per cent aqueous NH_3, R_F 0.28; (*c*) 1-butanol-

[2] All quantitative elemental analyses were supplied by Galbraith Laboratory, Knoxville, Tennessee.

[3] Attaclay is a hydrated aluminum silicate sold by Attapulgus Minerals and Chemicals Corporation, 210 West Washington Square, Philadelphia, Pennsylvania.

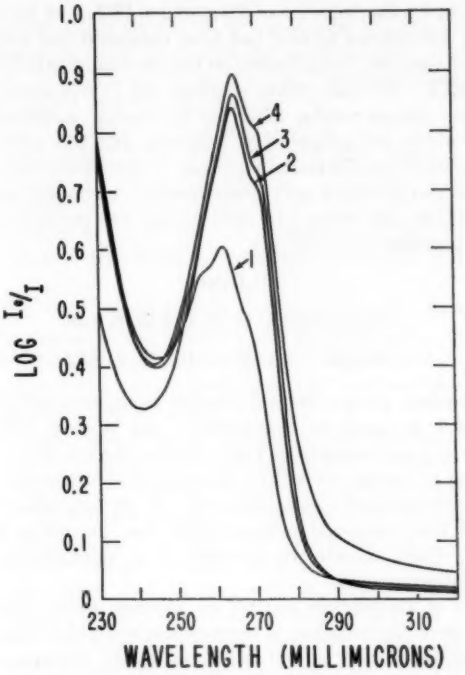

FIG. 1. Ultraviolet absorption spectra of 1, nicotinamide; 2, N^1-methylnicotinamide chloride; 3, N^1-(2-hydroxyethyl)nicotinamide chloride; 4, N^1-(2-hydroxyethyl)nicotinamide chloride (aqueous solution treated with 2.5 N NaOH at room temperature for 2 hours; neutralized with HCl). The concentration of all compounds was 2×10^{-4} M, in 0.01 M phosphate buffer, at pH 7.0.

acetic acid-H_2O (100:21:50), R_F 0.36; (*d*) phenol saturated with H_2O, R_F 0.55. The capacity of *N*-alkylpyridinium salts to yield the corresponding alkylamines upon being refluxed with strong alkali has been reported (21).

As would be expected, the amide nitrogen of the N^1-(2-hydroxyethyl)nicotinamide[4] could be quantitatively recovered as ammonia.

As shown in Fig. 1, the absorption peak for nicotinamide is 262 mμ, whereas that for N^1-(2-hydroxyethyl)nicotinamide and N^1-methylnicotinamide is 265 mμ. On a molar basis, the absorption curves for the two latter compounds are considerably higher than that for nicotinamide. The similarity in the ultraviolet absorption curves suggested that the structure of the reaction product (N^1-(2-hydroxyethyl)nicotinamide) was similar to that of N^1-methylnicotinamide.

Instability of N^1-(2-hydroxyethyl)nicotinamide in Cold Alkali—A solution of 20 mg. of N^1-(2-hydroxyethyl)nicotinamide in 0.5 ml. of 1 N NaOH is at first yellow and becomes red in a few hours. Less than 0.5 per cent of the fluorometrically determined activity is recovered after this treatment. Paper chromatography of this alkaline solution in 1-propanol-H_2O (7:3) revealed a trace of the unchanged compounds as well as at least five new components: two yellow spots which fluoresced under ultraviolet irradiation, a colorless fluorescent spot, and two spots which strongly quenched the background fluorescence of the paper. Treatment with alkali of a sample of N^1-methylnicotinamide gave similar destruction and similar chromatographic results. The irreversible reaction of N^1-(2-hydroxyethyl)-nicotinamide in alkali hardly altered its ultraviolet absorption spectrum (Fig. 1).

[4] The word, chloride, will be omitted from the name of this compound even though in all cases that salt was the one isolated and used.

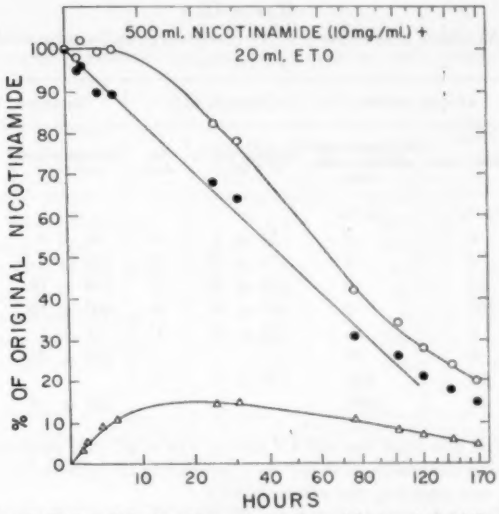

FIG. 2. Rate of destruction of nicotinamide treated with ethylene oxide at 5°. △, the per cent of original nicotinamide present in the reaction mixture as N^1-(2-hydroxyethyl)nicotinamide; ●, that present as unchanged nicotinamide; ○, the sum of the two.

TABLE II

Nicotinic acid activity of N^1-(2-hydroxyethyl)nicotinamide for Lactobacillus arabinosus

Sample No.	Contains		Nicotinic acid activity of sample as assayed
	Nicotinic Acid	N^1-(2-hydroxyethyl)nicotinamide	
	mμg./ml.	mμg./ml.	mμg./ml.
1	40	0	41
2	40	40	40
3	40	400	40
4	40	4000	42

The instability of N^1-(2-hydroxyethyl)nicotinamide at pH 11 to 12 is further seen from the kinetic data in Fig. 2. Initially the rate of accumulation of the primary reaction product follows the exponential rate of nicotinamide destruction. However, the pH rises from 6.5 to 11 during the first 30 minutes, and soon the initial product is degraded faster than it is formed.

Biological Activity of N^1-(2-hydroxyethyl)nicotinamide—The data in Table II indicate that N^1-(2-hydroxyethyl)nicotinamide has no growth-promoting nor antagonistic action for *L. arabinosus* when added to a medium containing suboptimal amounts of nicotinic acid. Additional tests of the biological activity of the compound were carried out with day-old chicks maintained on a nicotinic acid-deficient ration. Statistical evaluation of the 4-week weights (Table III) indicates that N^1-(2-hydroxyethyl)nicotinamide does not replace nor act as a metabolic antagonist for nicotinic acid in the chick.

Reaction of Ethylene Oxide with Nicotinic Acid—Of nicotinic acid (sodium salt) 81 mmoles in 40 ml. aqueous solution were treated with ethylene oxide, and the pH was maintained between

TABLE III

Nicotinic acid activity of N^1-(2-hydroxyethyl)nicotinamide for chicks

Addition to diet*		Experiment 1†		Experiment 2†	
Nicotinic Acid	N^1-(2-hydroxyethyl)nicotinamide	Average weight at 4 wks.‡	No. dead	Average weight at 4 wks.‡	No. dead
mg./kg.	*mg./kg.*	*gm.*		*gm.*	
0	0	54 ± 2	4	82 ± 8	2
5	0	79 ± 10	2	101 ± 8	0
10	0	133 ± 13	0	136 ± 16	1
100	0	283 ± 9	0	305 ± 10	0
0	10	73 ± 8	1	74 ± 4	2
0	20			103 ± 25	3
10	500	104 ± 6	1		
10	1000			129 ± 10	1

* The diet used was the C2 diet of Fox *et al.* (22) modified by reducing the casein to 15 per cent, increasing the gelatin to 13 per cent, and omitting the nicotinic acid.

† For each experiment, 6 female New Hampshire chicks were started on each regimen at 1 day of age.

‡ ± values indicate standard error.

TABLE IV

*Effect of temperature on rate of ethylene oxide destruction of nicotinic acid and nicotinamide activity**

Reaction temperature (°C.)	Per cent destroyed† after reaction period indicated			
	Nicotinic Acid		Nicotinamide	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
−20	0	10	4	18
5	11	41	7	32
24	41	89	24	70
37	73	99	47	90

* 1.0 ml. of ethylene oxide added to 100 ml. of 0.1 M aqueous vitamin solutions. Reactions stopped by adding 10 moles of NaCl and 10 moles of acetic acid per mole of ethylene oxide, then placing in a vacuum for 30 minutes.

† Determined microbiologically.

TABLE V

*Effect of pH on rate of ethylene oxide destruction of nicotinic acid and nicotinamide activity**

Reaction pH	Per cent destroyed after 6 hrs.†	
	Nicotinic Acid	Nicotinamide
4.0	42	58
6.0	74	53
8.0	90	73

* 2.0 ml. of ethylene oxide added initially per 100 ml. of 0.01 M aqueous solutions of the vitamin at 24°; ethylene oxide gas bubbled through solutions during the 6 hours, and pH was maintained by addition of 0.05 N HCl.

† Determined microbiologically.

7 and 11 by the dropwise addition of 2 N HCl. At the end of 7 hours, 100 mmoles of acid had been consumed and further pH change was slow, being limited to the reaction of ethylene oxide with HCl. The pale yellow solution (pH 7) was concentrated, and the viscous residue taken up in absolute methanol. The NaCl which precipitated was removed, and the product was precipitated from the methanol filtrate by the addition of acetone. It was recrystallized once from absolute methanol and once from 95 per cent ethanol to yield 5.6 gm. (46 per cent yield) of white platelets, m.p. 173° (d).

$C_8H_9NO_2$

Calculated: C 57.50, H 5.43, N 8.38

Found: C 57.49, H 5.37, N 8.22

This product gives a neutral reaction in aqueous solution, decomposes in alkali to ethanolamine and a deep red-colored product(s), and contains no ionic chloride ($AgNO_3$ test). All of the data are consistent with its proposed structure as the betaine of N^1-(2-hydroxyethyl)nicotinic acid. A strongly alkaline solution of this compound reduces silver ions, depositing a silver mirror, which indicates the presence of an aldehyde among its degradation products.

Effect of Temperature and pH on Reaction Rates—Tables IV and V show the influence of temperature and pH on the rate of vitamin destruction, as well as illustrating the comparative rate of reaction of ethylene oxide with nicotinamide and nicotinic acid.

With the exception of the reactions carried out at pH 4 (Table V), nicotinic acid has been consistently more labile to ethylene oxide treatment than nicotinamide. Table VI shows this difference in lability when the two compounds are treated after admixture with starch. This table also shows the extent of destruction of nicotinic acid activity in some natural products after treatment with ethylene oxide.

DISCUSSION

The present evidence suggests that when ethylene oxide reacts with nicotinic acid or nicotinamide, the hydroxyethyl radical adds to the ring nitrogen. The rate of product formation depends partly on the pH of the reaction mixture. At pH values below 7, the rate is much slower than at higher values. These findings suggest that it is the tertiary amine base and not its acid cation which participates in the reaction.

The reaction of ethylene oxide with tertiary nitrogen requires the presence of an available proton. Since water facilitates proton reactions, this may explain the enhancing effect of small amounts of moisture on the effectiveness of ethylene oxide fumigation procedures (23) and this, in turn, may partially determine the rate of destruction of nutrients during the fumigation of foods (3, 5).

At pH 6 or above, nicotinic acid reacts with ethylene oxide faster than does nicotinamide. If the rate of reaction of these two compounds were dependent only upon the degree of protonization, then the reverse of this should occur. Some factor other than protonization must determine the relative rates. One of these factors, might be the basicity of the ring nitrogen with the more basic group reacting faster.[5] The carboxyl group probably does not depress the basicity of the ring nitrogen to the same

[5] This was suggested by Dr. Louis Cohen.

degree as the amide group. If this is so, the pK_b for nicotinic acid should be lower than that for nicotinamide. The data presented by Hundley (24) on the pK_b values for these compounds conform with this hypothesis.

With nicotinamide, the reaction product was N^1-(2-hydroxyethyl)nicotinamide. This compound is rapidly changed above pH 11, forming a variety of substances. The reaction product, N^1-(2-hydroxyethyl)nicotinamide, has no biological activity for microorganisms or chicks, which is consistent with the inactivity of nicotinic acid derivatives in which the ring nitrogen is methylated.

In 1954, N^1-(2-hydroxyethyl)nicotinamide was synthesized from nicotinamide and ethylene chlorohydrin (25). The reported melting point was 182°. The melting point of the product isolated in the present study was 196° and was unchanged after repeated recrystallizations.

Each of the vitamins which is labile to treatment with ethylene oxide (2) contains a tertiary heterocyclic nitrogen. Since tertiary amines are even more reactive than primary or secondary amines in the presence of epoxides (26), one might speculate that the tertiary nitrogen is the common site for attack by ethylene oxide in all of these compounds. Preliminary evidence indicates that *N*-alkylation does occur during the destruction of pyridoxine, since a solution of pyridoxine became alkaline on treatment with ethylene oxide. The resulting solution failed to exhibit the typical pyridoxine color with the Gibbs Reagent (*N*,2,6-trichloro-*p*-benzoquinone imine) (27). The methiodide of pyridoxine also fails to give this test, probably because the strong inductive effect of the quaternary nitrogen deactivates the adjacent 6 position in the ring, thus preventing the electrophilic coupling. The ethylene oxide-pyridoxine reaction product has not been further purified, however. Folic acid and riboflavin, which are also labile to treatment with ethylene oxide, show a marked intensification of color when their solutions are treated. The most apparent sites of attack of ethylene oxide with the chromophoric groups of these vitamins are the tertiary nitrogens.

In this study no attempt was made to duplicate commercial procedures used in sterilizing foods. Our primary interest has been to study the reaction of the gas with some of the compounds (vitamins and amino acids) which previous work has shown were inactivated during treatment with ethylene oxide. It is likely that, during gaseous fumigation, the same reaction product is formed from nicotinamide as that isolated in this study. Evidence for this was the finding that when nicotinamide is dispersed in starch and treated with ethylene oxide, a compound was formed which acted similarly to N^1-(2-hydroxyethyl)nicotinamide in that it did not fluoresce until treated with a ketone in the presence of alkali, this behavior being typical of quaternary pyridinium compounds with a side chain CO—NHR in the β position. Nicotinamide does not fluoresce under these conditions. In the ethylene oxide-treated sample, 80 per cent of the nicotinamide which had been destroyed (as determined by microbiological assay) was recovered in the form of N^1-(2-hydroxyethyl)nicotinamide (as determined by the fluorometric assay).

Some commercial methods for the ethylene oxide sterilization of foods may not destroy vitamins to the same extent as the procedure used in our laboratories. This is shown by the report (28) that one commercial method destroyed less than 15 per cent of the nicotinic acid in yeast and in a commercial rat diet. Our results (Table VI) indicate a loss of 22 per cent of the nicotinic acid activity in yeast and from 53 to 69 per cent of the activity in two commercial animal diets. A number of factors undoubtedly determine the degree of nutrient destruction produced by ethylene oxide. These include moisture content and reaction temperature. Another factor is the relative amount of the nicotinic acid which is present as the nucleotide in the sample being fumigated. Our studies with DPN show that the nicotinamide therein is completely stable to treatment with ethylene oxide, as determined by microbiological assay.

TABLE VI

*Destruction of nicotinic acid activity by ethylene oxide fumigation**

Sample	Nicotinic acid activity†		Per cent destruction
	Before ethylene oxide	After ethylene oxide	
	μg./gm.	μg./gm.	
Pork liver (desiccated)	458	302	34
Brewers' yeast	471	366	22
Hunt Club Stock Diet	32	10	69
Purina chow	89	42	53
Enriched white flour	32	5	84
Nicotinamide + starch	2080	1200	42
Nicotinic acid + starch	2260	123	95

* Aliquots (100 gm.) of each sample fumigated, five at a time, in Petri dishes placed into a 10-l. desiccator with 100 ml. of liquid ethylene oxide. Fumigated for 18 hours at room temperature. For additional details see reference (2).

† Determined microbiologically.

SUMMARY

Ethylene oxide reacts with nicotinamide in aqueous solution at 25° to yield, after acidification with HCl, N^1-(2-hydroxyethyl)-nicotinamide chloride. This compound is unstable in alkali and possesses no nicotinic acid activity for *L. arabinosus* or for the chick; neither does it function as a nicotinic acid antagonist. It can be quantitatively determined by the fluorometric N^1-methylnicotinamide assay.

By a similar reaction, nicotinic acid is converted to the betaine of N^1-(2-hydroxyethyl)nicotinic acid by ethylene oxide.

The rate of reaction increases with increasing pH and temperature; at the higher pH reactions the initial products are unstable. There is a marked variation in the lability to ethylene oxide action of the nicotinic acid activity of various natural products. The nicotinamide of DPN is not affected, which probably explains the stability of the nicotinic acid activity in some natural products. Under most conditions, nicotinic acid is more labile to treatment with ethylene oxide than is the amide.

The relation of these findings to commercial ethylene oxide fumigation and to other vitamin-ethylene oxide reactions is discussed.

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REFERENCES

1. Windmueller, H. G., and Bakerman, H., *Federation Proc.*, **17**, 338 (1958).
2. Bakerman, H., Romine, M., Schricker, J. A., Takahashi, S. M., and Mickelsen, O., *J. Agri. Food Chem.*, **4**, 956 (1956).
3. Mickelsen, O., *J. Am. Dietet. Assoc.*, **33**, 341 (1957).
4. Windmueller, H. G., Ackerman, C. J., and Engel, R. W., *J. Nutrition*, **60**, 527 (1956).
5. Windmueller, H. G., Ackerman, C. J., and Engel, R. W., *J. Biol. Chem.* **234**, 895 (1959).
6. Lohmann, H., *J. prakt. Chem.*, **153**, 57 (1939).
7. Eastham, A. M., Darwent, B. deB., and Beaubien, P. E., *Can. J. Chem.*, **29**, 575 (1951).
8. Eastham, A. M., and Darwent, B. deB., *Can. J. Chem.*, **29**, 585 (1951).
9. Eastham, A. M., *J. Chem. Soc.* 1936 (1952).
10. Dann, W. J., and Satterfield, G. H., *Biological symposia, Vol. XII*, The Ronald Press Company, New York, 1947, p. 188.
11. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).
12. Knorr, L., *Ber. deut. chem. Ges.*, **32**, 729 (1899).
13. Sidgwick, N. V., *Organic chemistry of nitrogen*, Clarendon Press, New York, 1937, p. 524.
14. Beilstein, F. K., *Handbuch der Organischen Chemie, Vol. IV* Edward Brothers, Inc., Ann Arbor, Michigan, 1944, p. 285.,
15. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M., *J. Biol. Chem.*, **191**, 447 (1951).
16. Burton, R. M., and San Pietro, A., *Arch. Biochem. Biophys.*, **48**, 184 (1954).
17. Kodicek, E., and Reddi, K. K., *Nature*, **168**, 475 (1951).
18. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).
19. Critchfield, F. E., and Johnson, J. B., *Anal. Chem.*, **29**, 797 (1957).
20. Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1939).
21. Mosher, H. S., in R. C. Elderfield (Editor), *Heterocyclic compounds, Vol. I*, John Wiley and Sons, Inc., New York, 1950, p. 425.
22. Fox, M. R. S., Ortiz, L. O., and Briggs, G. M., *J. Agr. Food Chem.*, **3**, 436 (1955).
23. Phillips, C. R., and Kaye, S., *Am. J. Hyg.* **50**, 279 (1949).
24. Hundley, J. M., in W. H. Sebrell and R. S. Harris (Editors), *The vitamins, Vol. II*, Academic Press, Inc., New York, 1954, pp. 464, 472.
25. Drefahl, G., and Koenig, K., *Chem. Ber.*, **87**, 1628 (1954).
26. Ross, W. C. J., *Advances in Cancer Research*, **1**, 429 (1953).
27. Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, **61**, 1237 (1939).
28. Oser, B. L., and Hall, L. A., *Food Technol.*, **10**, 175 (1956).

Reaction of Ethylene Oxide with Histidine, Methionine, and Cysteine*

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Since it was shown (2) that the treatment of casein with ethylene oxide could diminish the biological availability of the histidine and methionine of this protein, it became of interest to determine whether other proteins were affected in the same way and to elucidate the nature of the chemical reactions involved. Particular interest in such reactions derives from the use of ethylene oxide as a fumigant in the food industry (3, 4) and from the inability of an earlier study (5) of proteins treated with this reagent to account for the interaction with histidine and methionine. The reactions of ethylene oxide with available sulfhydryl, hydroxyl, carboxyl, and primary amino groups of proteins have been documented (5).

The destructive action of ethylene oxide on histidine and methionine has now been observed with two other proteins, and lysine appears to be another amino acid which is altered by ethylene oxide. Chemical justification for these observations was sought. Initially, model systems were investigated in which the individual amino acids or amino acid derivatives were treated with ethylene oxide in aqueous solution. In the case of histidine, the studies have been extended to intact protein. Evidence is presented that the nitrogen atoms of the imidazole ring and the sulfur of methionine undergo hydroxyethylation under mild conditions in the presence of ethylene oxide, imidazole yielding the 1,3-bis-(2-hydroxyethyl)imidazolium ion and *N*-acetylmethionine being converted to *S*-(2-hydroxyethyl)-*N*-acetylmethionine, thetin. The double alkylation of the mercapto group of cysteine also can result in formation of a sulfonium derivative.

EXPERIMENTAL

Materials and Methods—The technique for treating solutions with gaseous ethylene oxide has been previously described (6). The process is conducted at room temperature and in the presence of a large excess of the gas.

The moisture content of commercial proteins was determined gravimetrically after the preparations had been dried for 48 hours in a forced-draft oven at 80°. The same treatment was used in preparing the moisture-free samples. The protein samples were treated as previously described (2). After exposure to ethylene oxide, the samples were hydrolyzed in 2.5 N HCl at 121° for 12 hours, and microbiological amino acid assays were conducted on the hydrolysates.

N-acetylmethionine was prepared as described by Kolb and Toennies (7). A Beckman model M2 pH meter with glass electrodes was used in preparing the titration curves. Primary amino groups were determined by the manometric nitrous acid method of Van Slyke (8).

Imidazole was purchased from Eastman Chemical Corporation and all amino acids and proteins from Nutritional Biochemicals. They were used without further purification. Quantitative elemental analyses were supplied by Galbraith Laboratories, Knoxville, Tennessee.

RESULTS

Amino Acids of Ethylene Oxide-treated Proteins—The diminution of biologically-available histidine, methionine, and lysine of three proteins after treatment with ethylene oxide is shown in Table I. That the decrease in amino acid content was not the result of a growth antagonist in the treated proteins was demonstrated by assaying each hydrolysate in duplicate at five different concentrations.

The inability of the treated protein samples to support the growth of weanling rats was demonstrated by incorporating them at a level of 9 per cent into a purified diet (2) as the sole source of amino acid nitrogen. The inhibition of growth was completely reversed in each case by dietary supplements of DL-histidine-HCl, DL-methionine, and L-lysine-HCl, or in some cases only one

Table I

Apparent decrease of histidine, methionine, and lysine in three proteins treated with ethylene oxide

Protein	Moisture	Treatment	Histidine*	Methionine*	Lysine*
	%	hrs.	mg./gm. dry protein†		
Casein (Labco)	0	0	26.1	26.5	72.4
	0	24	18.9 (28)	21.8 (18)	67.6 (7)
	10	24	11.1 (57)	14.3 (46)	55.6 (23)
Lactalbumin	0	0	14.7	17.9	76.6
	0	24	8.0 (46)	13.8 (23)	52.7 (31)
	10	24	2.5 (83)	8.0 (55)	55.0 (28)
Egg albumin	0	0	19.7	29.2	56.7
	0	24	17.6 (11)	27.3 (6)	53.6 (5)
	11.8	24	5.7 (71)	19.5 (33)	4.8 (92)

* Numbers in parentheses indicate the per cent decrease after treatment with ethylene oxide.

† Determined microbiologically, with *Leuconostoc mesenteroides* P-60.

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or two of these amino acids, depending on the protein and the level of moisture content during the treatment. Thorough drying of the proteins before treatment reduced the damaging effect of the ethylene oxide.

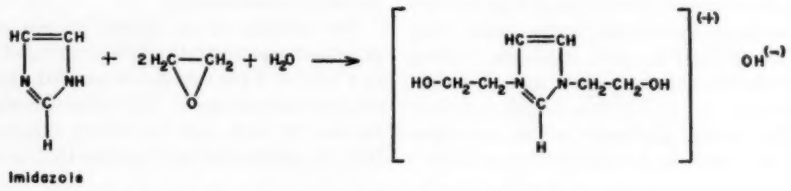
Reaction of Ethylene Oxide with Imidazole—During exposure to ethylene oxide, an aqueous solution of imidazole became strongly alkaline, suggesting the production of a quaternary ammonium group in analogy with the behavior of pyridine derivatives when treated with ethylene oxide (6). The product was isolated as follows. Imidazole, 70 mmoles, and HCl, 140 mmoles, in 20 ml. of H_2O were treated with gaseous ethylene oxide for 72 hours. The final pH was 9.5. The solution was acidified with HCl, concentrated to a syrup under vacuum, diluted with 20 ml. of hot absolute ethanol, and filtered. Acetone was added dropwise to the point of incipient cloudiness and crystallization allowed to proceed overnight at −2°. The product was recrystallized twice from ethanol-acetone to yield fine, white, highly deliquescent needles which were dried in a vacuum over H_2SO_4. Phosphorus pentoxide could not be used since the product sublimed somewhat under vacuum and was dehydrated and polymerized on the surface of this desiccant. The product was too deliquescent for convenient melting point determination. Elementary analysis was

$C_7H_{13}N_2O_2Cl$

Calculated: C 43.58, H 6.81, N 14.55, Cl 18.42

Found: C 43.36, H 6.99, N 14.26, Cl 18.18

From the empirical formula, it was evident that 2 moles of ethylene oxide had been added to the imidazole molecule. When no acidic hydrogen could be titrated with sodium methoxide (ruling out the possibility that the compound was a hydrochloride) the reaction was presumed to have occurred as shown in Scheme I.



SCHEME I. 1,3-bis-(2-hydroxyethyl)imidazolium hydroxide

This product was converted to the 1,3-bis-(2-hydroxyethyl)-imidazolium chloride (Compound I) upon the addition of HCl.

Additional evidence for this structure was obtained by degrading Compound I in 25 per cent KOH (weight for weight). Pinner and Schwarz (9) have demonstrated that 1,3-dialkyl imidazoles yield the corresponding primary alkyl amines by such treatment, *e.g.* ethanolamine from the proposed Compound I. A mmole of Compound I (as determined by inorganic chloride analysis (10)) was refluxed for 24 hours in 25 per cent KOH (weight for weight) and then distilled into dilute acid. By the method previously described (6), ethanolamine was identified in the distillate by paper chromatography and quantitatively determined by Van Slyke amino nitrogen analysis and by periodate oxidation to formaldehyde, which was colorimetrically determined with chromotropic acid. The only amine found was ethanolamine in 80 per cent of the theoretical yield of 2 mmoles.

Unlike imidazole, Compound I gives no color when coupled with diazotized sulfanilic acid in the quantitative histidine assay method of McPherson (11), thereby making this a suitable method for following the reaction of ethylene oxide with imidazole and some of its derivatives, *e.g.* histidine.

Reaction of Ethylene Oxide with Histidine—The treatment of a solution of histidine-HCl for 24 hours with ethylene oxide yielded a product which resisted all attempts at crystallization. The following data were obtained by analysis of the product in solution.

The product formed no colored complex with diazotized sulfanilic acid, implying hydroxyethylation of the imidazole nitrogens as in the case of ethylene oxide-treated imidazole. This idea was supported by the recovery of ethanolamine (63 per cent of theory) from a 25 per cent KOH (weight for weight) hydrolysate of the product. The product did not react with ninhydrin and a calculated 0.02 mmoles of the product in solution was analyzed for primary amino nitrogen by the Van Slyke manometric method. Within the experimental error of the method (±2 per cent), no nitrogen could be measured. This is taken as evidence that the primary amino group has been alkylated as would be expected (5, 12). Unlike histidine, the product formed an immediate water-insoluble precipitate with ammonium reineckate at acid pH, further support for the presence of the quaternary ammonium group.

Chemical Detection of Ethylene Oxide-Imidazole Reaction in Proteins—Table II shows the decrease in apparent histidine of casein samples of various moisture content treated with ethylene oxide. Amino acids other than histidine may contribute a small amount of color in this assay (13), so the values listed in the table represent the apparent histidine content. Samples 1 and 4 were also assayed for histidine microbiologically; the ethylene oxide-treated sample had 71 per cent less histidine than the untreated control.

TABLE II

Decrease of chemically determined histidine of casein treated with ethylene oxide

Sample	Moisture	Treatment	μg. histidine* / mg. nitrogen	Per cent decrease after treatment
	%	*hrs.*		
1	0	0	312	0
2	0	12	215	31
3	9.5	12	120	60
4	9.5	24	94	70

* After treatment, the samples were hydrolyzed in 2.5 N HCl for 12 hours at 121°, neutralized with NaOH, decolorized with charcoal (Norit), and assayed for histidine by the colorimetric method of McPherson (11), standardized with DL-histidine-HCl. Nitrogen was determined by the standard Kjeldahl method.

There is some evidence that in native protein, imidazole groups may be protected from reaction with ethylene oxide. No diminution in histidine (chemical assay) could be detected in a sample of crystalline chymotrypsin (Nutritional Biochemicals) which had been treated in aqueous solution with an excess of ethylene oxide for 5 hours, although the proteolytic activity (14), as measured on a casein substrate, had been reduced by about 30 per cent. Denaturing the chymotrypsin in 8 M urea apparently permitted reaction with the imidazole groups; subsequent treatment with ethylene oxide reduced the color in the histidine assay by at least 60 per cent.

Reaction of Ethylene Oxide with Methionine—The reaction of methionine with ethylene oxide yields a product which has resisted crystallization; therefore all evidence concerning its structure was obtained by analysis of solutions of methionine after treatment with ethylene oxide for 24 hours, followed by removal of excess reagent under vacuum. The product gives no color in the standard McCarthy-Sullivan nitroprusside assay (15) for methionine, suggesting alteration of the thioether group. Unlike methionine, the product forms a water-insoluble complex with the reineckate ion and with phosphotungstic acid. This behavior is typical of sulfonium derivatives of methionine (16). The solubility of the reineckate in dilute alkali is taken as evidence that the carboxyl group of the methionine remained unesterified. Primary amino groups had been completely alkylated as indicated by the failure of the product to react with ninhydrin and the failure of a calculated 0.02 mmole of the product to yield nitrogen by the Van Slyke manometric technique.

Ethylene Oxide Treatment of N-acetylmethionine—To study more directly the reaction of ethylene oxide with the thioether group, *N*-acetylmethionine was chosen as reactant. The pH of an aqueous solution of *N*-acetylmethionine shifted from 2 to 9 and the reaction product obtained is soluble in water and ethanol, but insoluble (separates as an oil) in acetone and hexane. Repeated attempts at crystallization have failed. Like ethylene oxide-treated methionine, the product yields a water-insoluble, acetone-soluble phosphotungstate. Since the formula of the phosphotungstate would be difficult to establish, the ratio of C:N:S was calculated from the elementary analysis:

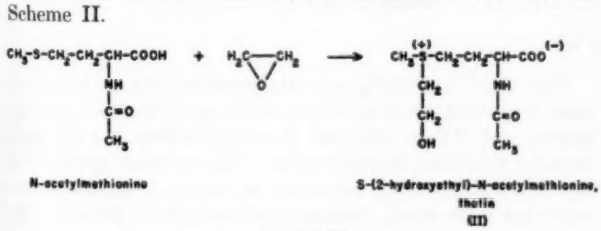
$C_9H_{17}NO_4S$-phosphotungstate

Found: C 6.53, N 0.86, S 2.11

The ratio of C:N:S was calculated to be 8.91:1:1.07 which reflects the addition of 1 mole of ethylene oxide to each mole of *N*-acetylmethionine.

The titration curve of the product is shown in Fig. 1. The compound is slightly alkaline in solution and has two titratable groups of pK′ approximately 2.9 and 12.1. It is apparent from the titration curve that the weak alkalinity of the product is not the result of esterification but that a basic group has been produced by the treatment with ethylene oxide.

All of the data are consistent with the formulation shown in Scheme II.



SCHEME II

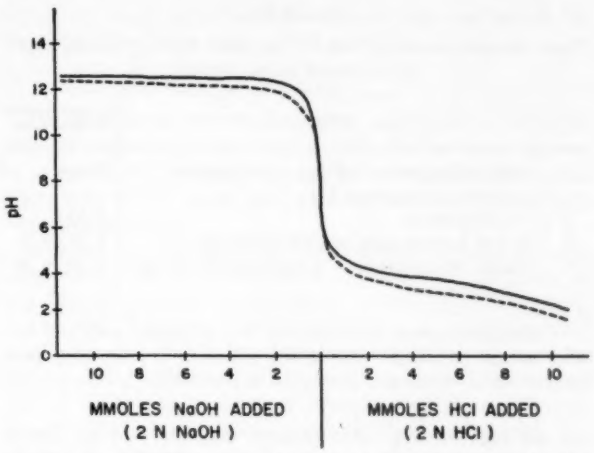


FIG. 1. Titration curve of 10.5 mmoles of *N*-acetylmethionine in 50 ml. of H_2O. Before treatment, pH 2.0 (——). After treatment with ethylene oxide for 24 hours, pH 9.0 (- - - -).

According to Toennies and Kolb (17), methionine sulfonium salts often are more easily crystallized than the corresponding *N*-acetyl derivatives. Therefore, in an effort to obtain a crystallizable product, the thetin was subjected to the hydrolytic activity of a preparation of acylase (18). Very little or no hydrolysis occurred, even when the enzyme was used at a concentration 10 times that which hydrolyzed almost completely a sample of *N*-acetyl-L-methionine.

Acid and Alkaline Hydrolysis of S-(2-hydroxyethyl)-N-acetylmethione—To gain additional information about the structure and stability of this thetin, which to the authors' knowledge has not been previously described, acid and alkaline hydrolysis were studied in some detail, particularly with respect to the stability of the sulfonium group.

Even after autoclaving a solution of Compound II in 6 N HCl for 14 hours at 121°, a large proportion of the sulfonium groups was still intact, as indicated by the voluminous precipitate which resulted upon the addition of phosphotungstic acid. However, paper chromatography of the autoclaved mixture in 1-butanol: acetic acid:H_2O (100:21:50) revealed five ninhydrin-positive spots, only one of which appeared to be a sulfonium compound. Sulfonium compounds were detected by spraying the chromatograms with a methanolic solution of ammonium reineckate, flushing the papers with water to remove excess reineckate, and, after drying, detecting the sulfonium-reineckate complexes by their quenching of ultraviolet irradiation.

As expected (19), the sulfonium group is labile to alkali. Merely warming a solution of Compound II in 0.5 N NaOH on a steam bath for 4 hours completely destroyed any ability to form a precipitate with phosphotungstate. A disagreeable cabbage-like odor was evolved during the hydrolysis.

Subsequently, a solution containing 1 mmole per ml. of Compound II in 0.5 N NaOH was slowly heated in a three-necked flask fitted with a nitrogen inlet, a thermometer, and a water condenser. The top of the condenser was connected to a series of three traps containing saturated aqueous $HgCl_2$ (19). Nitrogen was bubbled through the solution and traps during the gradual heating. At 90°, the nitrogen swept over a gas which formed a solid $HgCl_2$ adduct in the first trap. The gas was identified as CH_3SH by its odor and by the C:S:H ratio (1:1:3)

TABLE III

*Paper chromatography of NaOH hydrolysate of S-(2-hydroxyethyl)-N-acetylmethionine, thetin**

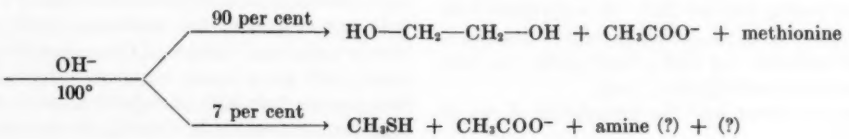
No.	Sample	R_F of ninhydrin-positive spots
1	*S*-(2-hydroxyethyl)-*N*-acetylmethionine, thetin (Compound II)	None
2	DL-methionine	0.51
3	NaOH hydrolysate of Compound II	0.23, 0.51
4	NaOH hydrolysate of Compound II + DL-methionine	0.24, 0.52

* Chromatographed on Whatman No. 1 paper, with a 1-butanol:acetic acid:H_2O (100:21:50) solvent; developed papers sprayed with 0.4 per cent ninhydrin in 1-butanol.

and the high melting point (greater than 300°) of the $HgCl_2$ adduct (20). After a few minutes, no more CH_3SH was evolved, and the first $HgCl_2$ trap was replaced by a trap containing saturated lead acetate to collect volatile mercaptans but not sulfides. The NaOH concentration of the thetin solution was increased to 1 N and the thetin was refluxed and aerated at 105° for 15 hours. No lead precipitate formed, but a compound accumulated in the second trap (saturated aqueous $HgCl_2$) and is thought to have been CH_3—S—S—CH_3, according to the C:S ratio (1:1) and melting point of its $HgCl_2$ adduct (found, 142–144°, uncorrected; literature, 147–148° (16)). The CH_3SH and CH_3—S—S—CH_3 accounted for only 7 per cent of the sulfur of the thetin although the sulfonium group was completely destroyed during the hydrolysis.

Analysis of the thetin solution before and after alkaline hydrolysis revealed that the alkali had liberated a compound which was oxidized to formaldehyde by alkaline periodate. The formaldehyde was determined colorimetrically with chromotropic acid (21). If this compound were ethylene glycol, a likely hydrolytic product of this thetin (19, 22) or any other compound which yields 2 moles of formaldehyde when oxidized with periodate, then, from the quantitative data, a yield of 0.9 mmole per mmole of thetin was obtained. Therefore methionine was suspected as another major hydrolytic product. For this reason, the hydrolyzed thetin solution was chromatographed, with the results shown in Table III. Methionine does appear to be a product. The other ninhydrin-positive compound has not been identified.

The alkaline hydrolysis of Compound II may be tentatively formulated as shown in Scheme III.



SCHEME III

Reaction of Ethylene Oxide with Cysteine-HCl—The reaction of an aqueous solution of cysteine-HCl (pH 1.0) with ethylene oxide produces a clear yellow-colored solution and elevates the pH to 8.5. The product, which could not be crystallized, yields an insoluble precipitate with phosphotungstic acid. This property is destroyed by heating the product to 100° in dilute alkali for a few minutes. As a result of the studies with methionine, these data are taken as evidence that 2 moles of ethylene oxide converted the mercapto group into a sulfonium group.

$$R—SH + 2C_2H_4O \xrightarrow{H_2O} R—\overset{+}{S}(CH_2—CH_2—OH)_2 + OH^- \quad (3)$$

The primary amino group of the cysteine had been completely alkylated by the ethylene oxide as indicated by the failure of the product to react with ninhydrin and the failure to yield nitrogen when analyzed (0.02 mmole of the product in solution) by the Van Slyke manometric procedure. Again, the final pH of the reaction solution is evidence that the carboxyl group of the cysteine had not been esterified. Esterification would have consumed another proton and a strongly alkaline solution would have been expected.

DISCUSSION

The reactions of ethylene oxide with histidine, methionine, cysteine, and with the vitamins of the pyridine family (6) all have certain features in common. In each case, the reaction involves electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, either nitrogen or sulfur. The products are all produced under mild conditions, and in each case they are highly polar compounds containing an "onium" group. Tertiary heterocyclic nitrogen appears to be especially easily hydroxyethylated, so that one might expect purines and pyrimidines to be likely reactants. The alkylating potential of ethylene oxide is in many ways similar to that of the sulfur and nitrogen mustards (23, 24), which can also alkylate the nitrogen atoms of imidazole and the sulfur of methionine (25, 26).

The applicability of the reactions reported in this paper to the safety of using ethylene oxide as a commercial fumigant in the food industry has been previously discussed (6, 27). The studies with fumigated proteins give qualitative evidence of the potential danger of fumigating proteinaceous foodstuffs with ethylene oxide. Reasons for the accelerating action of moisture in ethylene oxide-vitamin reactions were discussed previously (6). Similar reasoning would apply to the ethylene oxide-amino acid reactions.

The strong polarity of the ethylene oxide-amino acid products may help to explain the isoelectric and electrophoretic shifts of proteins after ethylene oxide treatment, as observed by Fraenkel-Conrat (5), whose methodology could not detect the specific reactions with the imidazole of histidine and the thioether group of methionine. From theoretical considerations, the involvement of imidazole group quaternization in these shifts has already been suggested by Alexander (23).

That the 1,3-bis-(2-hydroxyethyl)histidine has no biological value for the rat would have been anticipated from the report by Sakami and Wilson (28) that 1-methylhistidine is inert as a histidine substitute in this species. The apparent inertness of the *S*-(2-hydroxyethyl) sulfonium derivative of methionine is somewhat more novel, considering the report by Bennett (29)

that the methylsulfonium derivative will replace methionine for rats grown on a methionine-deficient diet.

The reaction product of ethylene oxide with lysine has not been identified. The decrease in microbiologically available lysine of ethylene oxide-treated Labco casein, lactalbumin, and egg albumin is influenced by the presence of water and possibly by the protein itself. These ethylene oxide-treated proteins were unable to support the growth of weanling rats when fed at a level of 9 per cent of the diet.[1] By supplementation experiments, Labco casein and lactalbumin were found to be deficient in histidine and methionine, and egg albumin deficient in histidine and lysine.[1] In analogy with the results with other amino acids, it is assumed that hydroxyethylation of one or both of the primary amino groups of lysine can account for the observed decrease in the availability of this amino acid.

The toxicity of subcutaneous injections of the ethylene oxide-cysteine-HCl reaction product in the rat was reported in an earlier paper (2). Now, with more information about the probable structure of the product, a relationship is suggested between this compound and a series of synthetic sulfoniums (30, 31) which act as spasmolytics, presumably by virtue of their antagonism to acetylcholine. The toxic effect of the cysteine derivative does appear to be mediated through the central nervous system. Death is preceded by a generalized flaccid paralysis and respiratory failure. The synthetic spasmolytics and the cysteine product have the following structure in common: $(R)_2S^+—CH_2—CH_2—O^-$. The relationship to acetylcholine is apparent. The product of the ethylene oxide treatment of methionine did not induce any toxic symptoms when injected into two rats at 2.5 times the LD_{50} of the cysteine-HCl product.

SUMMARY

The decrease in the microbiological availability of histidine and methionine in proteins treated with ethylene oxide appears to be correlated with the electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, particularly nitrogen and sulfur.

In model experiments conducted in aqueous solution at room temperature, ethylene oxide reacts readily with imidazole or histidine to yield the corresponding 1,3-bis-(2-hydroxyethyl)-imidazolium derivative. With methionine or *N*-acetylmethionine, ethylene oxide hydroxyethylates the sulfur to yield the corresponding sulfonium derivatives. Likewise, double alkylation of the mercapto group of cysteine produces a sulfonium group. The primary amino groups of these amino acids also become alkylated, but esterification of carboxyl groups does not seem to be involved.

The lability in alkali of the ethylene oxide-produced "onium" groups in these amino acids has been studied and some of the degradation products identified.

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REFERENCES

1. Windmueller, H. G., and Bakerman, H., *Federation Proc.*, **17**, 338 (1958).
2. Windmueller, H. G., Ackerman, C. J., and Engel, R. W., *J. Nutrition*, **60**, 527 (1956).
3. *Chemical Week*, Oct. 2, 1954, p. 96.
4. Whelton, R., Phaff, H. J., Mrak, E. M., and Fisher, C. D., *Food Inds.*, **18**, 23, 174, 318 (1946).
5. Fraenkel-Conrat, H., *J. Biol. Chem.*, **154**, 227 (1944).
6. Windmueller, H. G., Ackerman, C. J., Bakerman, H., and Mickelsen, O. *J. Biol. Chem.*, **234**, 889 (1959).
7. Kolb, J. J., and Toennies, G., *J. Biol. Chem.*, **144**, 193 (1942).
8. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).
9. Pinner, A., and Schwarz, R., *Ber. deut. chem. Ges.*, **35**, 2441 (1902).
10. Saifer, A., and Hughes, J., *J. Biol. Chem.*, **129**, 273 (1939).
11. McPherson, H. T., *Biochem. J.*, **36**, 59 (1942); **40**, 470 (1946).
12. Maekawa, K., and Kushibe, M., *Bull. Agr. Chem. Soc. Japan*, **20**, 106 (1956).
13. Howard, A. N., and Wild, F., *Biochem. J.*, **65**, 651 (1957).
14. Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947).
15. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
16. Lavine, T. F., and Floyd, N. F., *J. Biol. Chem.*, **207**, 97 (1954)
17. Toennies, G., and Kolb, J. J., *J. Am. Chem. Soc.*, **67**, 849 (1945).
18. Birnbaum, S. M., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **194**, 455 (1952).
19. Ingold, C. K., Jessop, J. A., Kuriyan, K. I., and Mandour, A. M. M., *J. Chem. Soc.*, 533, (1933).
20. Challenger, F., and Rawlings, A. A., *J. Chem. Soc.*, 868, (1937).
21. Critchfield, F. E., and Johnson, J. B., *Anal. Chem.*, **29**, 797 (1957).
22. Challenger, F., and Simpson, M. I., *J. Chem. Soc.*, 1591, (1948).
23. Alexander, P., *Advances in Cancer Research*, **2**, 4 (1954).
24. Ross, W. C. J., *Advances in Cancer Research*, **1**, 429 (1953).
25. Stein, W. H., and Moore, S., *J. Org. Chem.*, **11**, 681 (1946).
26. Fruton, J. S., Stein, W. H., and Bergmann, M., *J. Org. Chem.*, **11**, 559 (1946).
27. Mickelsen, O., *J. Am. Dietet. Assoc.*, **33**, 341 (1957).
28. Sakami, W., and Wilson, D. W., *J. Biol. Chem.*, **154**, 223 (1944).
29. Bennett, M. A., *J. Biol. Chem.*, **141**, 573 (1941).
30. Protiva, M., and Exner, O., *Chem. Abstr.* **49**, 198i (1955).
31. Stein, W. H., Moore, S., and Bergmann, M., *J. Org. Chem.*, **11**, 664 (1946).

[1] H. G. Windmueller, C. J. Ackerman, and R. W. Engel, unpublished data.

The Biosynthesis of *N*-Carbamylhydroxyaspartic Acid*

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The formation of hydroxyaspartic acid *in vitro* by a transamination reaction between glutamate and oxaloglycolate has been established by studies in this laboratory (2) as well as by the independent work of Garcia-Hernandez and Kun (3). The present report is concerned with the further metabolism of this amino acid. Experiments to be described show that hydroxyaspartate reacts with carbamyl phosphate in the presence of enzyme preparations from normal rat liver or Ehrlich's ascites cell carcinoma. Proof is presented that the product of this transcarbamylation reaction is *N*-carbamylhydroxyaspartate. Certain characteristics of the reaction are discussed.

EXPERIMENTAL

Enzyme Preparations—Two enzyme preparations were used in these studies. The first was prepared from Ehrlich's ascites cell carcinoma. The ascites cells[1] were obtained by administering to 5- to 6-week-old mice intraperitoneal injections of an isotonic NaCl suspension of cells (approximately 10^7 cells per mouse). The mice were killed by decapitation 6 to 7 days after transplantation. The ascites fluid was removed, centrifuged, and the supernatant fluid discarded. The cells were washed four times by resuspending them in 6 volumes of 0.154 M NaCl solution and recentrifuging. One volume of cells was homogenized with 3 volumes of 0.154 M KCl solution in a Waring blendor for 2 minutes. The homogenate was centrifuged at 12,000 × *g* for 30 minutes at 0°. The supernatant solution was the enzyme preparation used in all experiments with ascites cells.

The second source of enzyme was normal rat liver. Young rats weighing 50 to 70 grams were used. A homogenate of rat liver was prepared by blending 1 part by weight of tissue with 9 volumes of isotonic KCl in a Waring blendor for 2 minutes. The homogenate was centrifuged at 12,000 × *g* for 45 minutes at 0°. The supernatant solution was fractionated, in the cold, by the addition of 20 gm. of ammonium sulfate per 100 ml. of solution. The precipitated protein was recovered by centrifugation at 12,000 × *g* for 30 minutes at 0° and dissolved in cold glass-distilled water (3.0 ml. per gm. of tissue used). This fraction was used in all experiments in which rat liver was the source of the enzyme.

Protein was determined by the method of Lowry *et al.* (4).

Substrates—Synthetic and enzymatically formed hydroxyaspartic acid (*erythro*-β-hydroxy-L-aspartate (5)), was obtained as described previously (2). Hydroxyaspartate-2,3-C^{14} was synthesized from fumarate-2,3-C^{14} by the same procedure. It was isolated by chromatography on Dowex 1 formate. All samples of the amino acid gave theoretical nitrogen values on analysis. Uniformly labeled L-aspartate-C^{14} and the nonradioactive compound were commercial preparations. The carbamyl derivatives of aspartate and hydroxyaspartate were prepared from the corresponding C^{14}-labeled amino acids by the method of Nyc and Mitchell (6). Nonradioactive carbamyl phosphate, as the crystalline ammonium salt,[2] was generously provided by Dr. Robert Metzenberg of this department. The C^{14}-labeled compound was synthesized by the method of Jones *et al.* (7). The potassium cyanate-C^{14} used in this synthesis was prepared from urea-C^{14} as described by Williams and Ronzio (8). Carbamyl phosphate preparations were analyzed for the carbamyl group by enzymatic conversion to citrulline (9).

Ion Exchange Chromatography—Columns of Dowex 1-X10 formate, 200 to 400 mesh, of 1.0 cm. internal diameter and a resin bed length of 22.0 cm. were prepared and regenerated as described by Hurlbert *et al.* (10). The reaction mixtures were deproteinized with $HClO_4$ (final concentration 0.3 M) at 0°, centrifuged, and neutralized to pH 6 to 7 by the addition of 4 N KOH with phenol red as an internal indicator. The precipitated $KClO_4$ was removed from the cold solution by centrifugation, and aliquots of the supernatant solutions were passed through the columns. The latter were then washed with 15 ml. of water. In experiments in which radioactive carbamyl phosphate was used, the $C^{14}O_2$ formed from the unchanged carbamyl phosphate upon acidification was removed by aeration with CO_2 and N_2 before the neutralization step.

Elution System 1—The reservoir of the gradient elution apparatus contained 0.5 N formic acid, and the mixing vessel contained 250 ml. of water. The volume of the fractions was 2.2 ml. After 80 fractions had been collected, the 0.5 N formic acid was replaced by 2.0 N formic acid. The separation of a mixture containing 20 μmoles each of radioactive aspartate, hydroxyaspartate, and their carbamyl derivatives is shown in Fig. 1 below.

Elution System 2—In those experiments in which only one amino acid was used in the incubation flask, no separation of carbamyl derivatives was required. In such cases, the elution procedure was modified as follows. The amino acid was eluted by washing the column with 75 ml. of 0.25 N formic acid. The column was then placed on a fraction collector and the carbamylamino acid was eluted with 4.0 N formic acid. Fractions of 3.0 ml. were collected.

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[1] Obtained through the courtesy of Dr. A. G. LePage.

[2] R. L. Metzenberg, M. Marshall, and P. P. Cohen, to be published.

The elution system used is indicated with the individual experiments.

Incubation Conditions—Incubations were carried out in a water bath at 37°. Each flask contained diethanolamine hydrochloride buffer (pH 8.9) at a final concentration of 0.1 M and ethylenediaminetetraacetate at a final concentration of 0.001 M. Other conditions are reported with the individual experiments. It should be pointed out that at carbamyl phosphate concentrations of 0.05 M or higher, an appreciable nonenzymatic blank occurs (approximately 3 per cent). Therefore, the final concentration of this compound used in most of the studies was 0.005 M or less. It is important to stress that the samples, after deproteinization, were immediately washed into the columns. Under these conditions the blank was negligible. In all cases, controls were run simultaneously, and the values have been corrected for the nonenzymatic blank.

Determination of Carbamylamino Acids—The carbamylamino acids were determined by the colorimetric procedure of Koritz and Cohen (11) or by measurement of radioactivity when C^{14}-labeled substrates were used. The counting data presented here were determined by direct plating of the material on copper disks (12). The samples were counted in a model D 47 gas flow counter (Nuclear-Chicago) with the Micromil window. All assays for carbamylamino acids were made after chromatography of the reaction mixtures as outlined above.

RESULTS AND DISCUSSION

In the presence of carbamyl phosphate and hydroxyaspartate, radioactivity was found in the fractions corresponding to authentic carbamylhydroxyaspartate when either of these two substrates was labeled with C^{14} (Fig. 1). The fractions in question were not radioactive when C^{14}-hydroxyaspartate or C^{14}-carbamyl phosphate (not shown) was incubated separately with the enzyme preparation. Similar results were obtained with liver as the source of enzyme.

Individual fractions presumed to contain carbamylhydroxyaspartate were analyzed for carbamylamino acids by the colorimetric procedure. When these results were compared to those based on the specific activities of the substrates used, good agreement was obtained (Table I).

A series of incubations with radioactive substrates was made to accumulate sufficient material for identification purposes. The fractions corresponding to known carbamylhydroxyaspartate were pooled and lyophilized. The residue was dissolved in 3 ml. of water and the resulting solution (hereafter called Solution D) was used in the experiments described below. Solution D was found to contain 1.83×10^6 c.p.m. per ml. and 0.036 mmole per ml. of presumed carbamylhydroxyaspartate calculated from the specific activity of the substrates used. Aliquots of Solution D were chromatographed on paper with *n*-butanol:acetic acid:water (40:40:20) as the ascending solvent system. The dried paper sheet was cut into strips, and the radioactivity of the latter was determined in a gas flow counter. A single spot, with the same R_F (0.40 cm) as authentic carbamylhydroxyaspartate, was found. When the paper strip was sprayed with the *p*-dimethylaminobenzaldehyde reagent used by Fink *et al.* (13) for the detection of carbamylamino acids, a yellow spot developed which coincided with the C^{14}-labeled area. Paper chromatography of Solution D in three other solvent systems gave similar results.

The isolation of *O*-carbamyl-D-serine from the culture media of a strain of *Streptomyces* has been reported (14). The formation of *O*-carbamylhydroxyaspartate in this system was ruled out by the following findings. Aliquots (0.2-ml.) of Solution D did not give a positive ninhydrin reaction when analyzed by the method of Moore and Stein (15); in addition, no ninhydrin reacting material could be detected on the paper chromatograms described above. When 1 ml. of Solution D was applied to a 1 × 22-cm. column of Dowex 50, hydrogen form, and the column was washed three times with 20 ml. of water, all of the radioactivity was recovered in the water effluent.

Although the above experiments established that the *O*-carbamyl derivative of the amino acid was not the product of the transcarbamylation reaction, the possible formation of the disubstituted derivative *i.e.* *O*-carbamyl-*N*-carbamylhydroxyaspartate

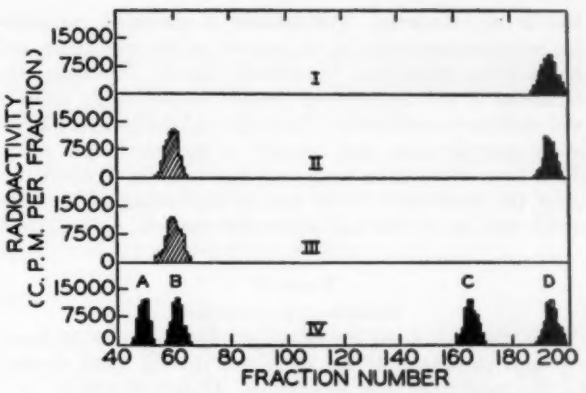

FIG. 1. Chromatography of reaction mixtures by Elution System 1 as described in the text. Substrates used were: I, carbamyl phosphate-C^{14} (25 μmoles per flask, 8080 c.p.m. per μmole) and nonlabeled hydroxyaspartate; II, hydroxyaspartate-C^{14} (8100 c.p.m. per μmole) and nonradioactive carbamyl phosphate (25 μmoles); III, hydroxyaspartate-C^{14} alone (8100 c.p.m. per μmole). Each flask contained 100 μmoles of hydroxyaspartate; 18 mg. of protein (ascites cells). Final volume 6.1 ml.; incubation time, 1 hour. (Values represented by diagonal bars have been divided by 10 to permit plotting of data.) IV, a mixture of 20 μmoles each of authentic samples of the following C^{14}-labeled compounds: *A*, aspartate; *B*, hydroxyaspartate; *C*, carbamylaspartate; *D*, carbamylhydroxyaspartate.

TABLE I

Determination of carbamylhydroxyaspartate by two assay procedures in fractions after column choromatography

The reaction system contained 100 μmoles hydroxyaspartate-C^{14} (5080 c.p.m. per μmole); 25 μmoles of carbamyl phosphate; 15 mg. protein (liver). Final volume 6.1 ml.; incubation time, 1 hour. Elution System 1.

Fraction No.	Carbamylhydroxyaspartate per fraction as determined by	
	Radioactivity	Colorimetric assay
	μmoles	*μmoles*
188	0.51	0.55
190	0.99	0.90
192	1.39	1.29
194	1.29	1.20
196	0.85	0.88
198	0.54	0.54

had to be considered. The amount of presumed carbamylhydroxyaspartate produced, as measured by the colorimetric and radioactivity procedures, consistently agreed. Therefore, the formation of the disubstituted product seemed highly unlikely and could be occurring only if the mono- and disubstituted derivatives gave the same color intensity in the colorimetric assay. Since none of the latter compound was available to check this point, the stoichiometry of the reaction was investigated. Equimolar amounts of the two substrates were incorporated into carbamylhydroxyaspartate (Table II). These results preclude the formation of the disubstituted derivative of the amino acid.

In view of the results outlined above, it was concluded that the product formed from carbamyl phosphate and hydroxyaspartate in this system is *N*-carbamylhydroxyaspartic acid.

The amount of carbamylhydroxyaspartate formed is proportional to the concentration of enzyme as shown in Fig. 2. Similar results were obtained with the enzyme preparation from ascites cells. The reaction was linear with time (Fig. 3).

Synthetic hydroxyaspartate, which is a mixture of four isomers, was used in many of the experiments reported in this paper. Since it was desirable to investigate the activity of the natural isomer in this system, hydroxyaspartate was prepared enzymatically from oxaloglycolate and glutamate and isolated as described previously (2). A comparison of activities with the isolated and synthetic acids is shown in Table III. It is evident that the isomer formed enzymatically is the reactive one in the transcarbamylation reaction.

The enzyme preparations used in these experiments are rich in the enzyme aspartic transcarbamylase. No attempt was made to separate the two activities. Preliminary inhibition studies (Table IV) show that, at equimolar concentrations of hydroxyaspartate and aspartate, carbamylhydroxyaspartate formation is decreased 50 per cent, and carbamylaspartate production is decreased 30 per cent from the amounts observed with the respective amino acids alone. It is known that hydroxyaspartate is a competitive inhibitor of aspartate for growth of *Escherichia coli* and *Leuconostoc mesenteroides* (16). Garcia-Hernandez and Kun, who used extracts of acetone powders of pig mitochondria,

TABLE II

Stoichiometry of reaction

Each flask contained 100 μmoles of amino acid; 25 μmoles of carbamyl phosphate; 6.0 mg. of protein (liver). Final volume, 6.1 ml.; incubation time, 30 minutes. Elution System 2.

Experiment No.	Specific activity of substrates		Total radioactivity incorporated into carbamylhydroxyaspartate	Carbamylhydroxyaspartate formed
	Carbamyl phosphate	Hydroxyaspartate		
	c.p.m./μmole		*c.p.m.*	*μmoles*
1	52,500	0	241,700	4.6
2	52,500	22,000	338,800	4.5
3	0	22,000	99,400	4.5

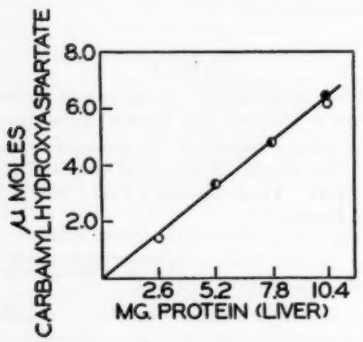

FIG. 2. Enzyme concentration curve for carbamylhydroxyaspartate formation. ○——○, radioactivity; ●——●, colorimetric assay. Each flask contained 100 μmoles of hydroxyaspartate-C^{14} (9700 c.p.m. per μmole); 25 μmoles of carbamyl phosphate. Final volume, 6.1 ml.; incubation time, 1 hour. Elution System 2.

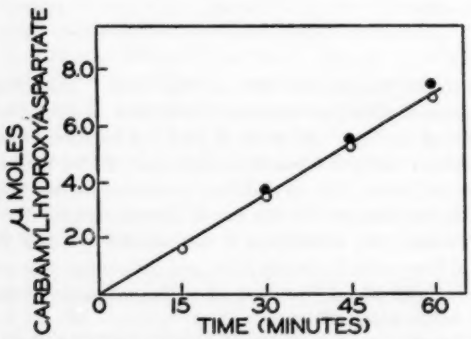

FIG. 3. Time curve for carbamylhydroxyaspartate formation. ○——○, radioactivity; ●——●, colorimetric assay. Each flask contained 100 μmoles of hydroxyaspartate-C^{14} (5080 c.p.m. per μmole); 25 μmoles of carbamyl phosphate; 14 mg. protein (ascites cells). Final volume, 6.1 ml. Elution System 2.

TABLE III

Carbamylhydroxyaspartate formation from isolated and synthetic hydroxyaspartic acids

Each flask contained 100 μmoles of carbamyl phosphate-C^{14} (8080 c.p.m. per μmole); amino acid as indicated; 16 mg. of protein (liver). Final volume 6.5 ml.; incubation time, 30 minutes. Elution System 2.

Amino acid	Carbamylhydroxyaspartate formed from	
	Isolated hydroxyaspartate	Synthetic hydroxyaspartate
μmoles	*μmoles*	*μmoles*
15	3.5	1.7
40	6.5	3.7
60	9.0	4.3
80	11.8	5.6

TABLE IV

Inhibition studies

Each flask contained 100 μmoles of carbamyl phosphate-C^{14} (8080 c.p.m. per μmole); amino acids as indicated; 14.5 mg. of protein (liver). Final volume, 6.5 ml.; incubation time, 30 minutes. Elution System 1.

Experiment No.	Amino acids incubated		Carbamylhydroxyaspartate formed	Carbamylaspartate formed
	Isolated hydroxyaspartate	L-Aspartate		
	μmoles	*μmoles*	*μmoles*	*μmoles*
1	80		13.3	
2	80	80	6.4	17.0
3		80		25.2

have demonstrated that hydroxyaspartic acid competitively inhibits the transamination of aspartate and α-ketoglutarate (3). These results suggest that the transcarbamylation of hydroxyaspartate observed in these studies may be due to a lack of specificity of the aspartic transcarbamylase. In this connection, it may be noted that, if carbamylhydroxyaspartate is further metabolized by the same metabolic pathway as carbamylaspartate, it should give rise to 5-hydroxyuridine, a known antimetabolite of nucleic acid synthesis (*cf.* (17)). Additional studies along these lines are in progress.

SUMMARY

The enzymatic reaction of hydroxyaspartic acid with carbamyl phosphate has been studied. *N*-carbamylhydroxyaspartate has been identified as the product of the reaction.

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REFERENCES

1. Sallach, H. J., *Federation Proc.*, **17**, 302 (1958).
2. Sallach, H. J., and Peterson, T. H., *J. Biol. Chem.*, **223**, 629 (1956).
3. Garcia-Hernandez, M., and Kun, E., *Biochim. et Biophys. Acta*, **24**, 78 (1957).
4. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
5. Sallach, H. J., *J. Biol. Chem.*, **229**, 437 (1957).
6. Nyc, J. F., and Mitchell, H. J., *J. Am. Chem. Soc.*, **69**, 1382 (1947).
7. Jones, M. E., Spector, L., and Lipmann, F., *J. Am. Chem. Soc.*, **77**, 819 (1955).
8. Williams, D. L., and Ronzio, A. R., *J. Am. Chem. Soc.*, **74**, 2407 (1952).
9. Grisolia, S., and Cohen, P. P., *J. Biol. Chem.*, **198**, 561 (1952).
10. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954).
11. Koritz, S. B., and Cohen, P. P., *J. Biol. Chem.*, **209**, 145 (1954).
12. Lowenstein, J. M., and Cohen, P. P., *J. Biol. Chem.*, **213**, 689 (1955).
13. Fink, R. M., McGaughey, C., Cline, R. E., and Fink, K., *J. Biol. Chem.*, **218**, 1 (1956).
14. Hagemann, G., Penasse, L., and Teillon, J., *Biochim. et Biophys. Acta*, **17**, 240 (1955).
15. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
16. Shive, W., and Macow, J., *J. Biol. Chem.*, **162**, 451 (1946).
17. Visser, D. W., in C. P. Rhoads (Editor), *Antimetabolites and cancer*, American Association for the Advancement of Science, Washington, 1955, p. 47.

Inhibition of *Saccharomyces cerevisiae* by *p*-Aminobenzoic Acid and Its Reversal by the Aromatic Amino Acids*

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It has been known for many years that *p*-aminobenzoic acid at high concentration is inhibitory to a variety of organisms, including rickettsiae (1), bacteria (2, 3), fungi (4), protozoa (5), and plants (6). The site of inhibition by *p*-aminobenzoic acid in most of these organisms has not been established. However, Davis (3) has shown that the bacteriostatic activity of *p*-aminobenzoic acid toward *Escherichia coli* (strain W) is reversed competitively by *p*-hydroxybenzoic acid. The inhibition was reversed also to a limited extent by shikimic acid and by 5-dehydroshikimic acid. Reversal by *p*-hydroxybenzoic acid of the rickettsiostatic action of *p*-aminobenzoic acid has been reported (7, 8). The present communication wll report that the aromatic amino acids, but not *p*-hydroxybenzoic acid or shikimic acid, exert a reversing effect on inhibition of *Saccharomyces cerevisiae* by *p*-aminobenzoic acid, and that shikimic acid accumulates in the culture medium in the presence of *p*-aminobenzoic acid.

EXPERIMENTAL

Materials and Methods

p-Hydroxybenzoic acid and *p*-aminobenzoic acid were obtained from the Matheson Company, and shikimic acid from the California Foundation for Biochemical Research. Other compounds were obtained from either the Nutritional Biochemicals Corporation or other standard commercial sources.

The strain of yeast used in this investigation, *S. cerevisiae* F.B. 7754, was isolated originally from Fleischmann bakers' yeast (9), and is carried on glucose-yeast extract-agar slants. The assay procedure and medium were modifications of those described previously by Snell *et al.* (9, 10) for the assay of biotin. The basal medium was supplemented with 100 mg. of L-glutamic acid and 0.02 μg. of biotin per l., and adjusted to a final pH of 4.5.[1] Addenda to the basal medium were adjusted to pH 4.5[1] and suitable aliquots were diluted to 1 ml.[2] with distilled water in 15 × 100-mm. ignition tubes. The tubes were covered with a clean towel and sterilized by steaming for 5 minutes.[3] When the tubes had cooled, 5 ml. of the basal medium (previously sterilized), containing 0.01 mg. (wet weight) of suspended yeast, were dispensed into each tube. The tubes were incubated at 30° for approximately 16 hours. Growth response was measured with a thermocouple-turbidimeter (11) adjusted so that it read 0 with distilled water and 100 with an opaque object.

The detection of accumulated aromatic precursors, and quantitative assays for shikimic acid, were performed with *E. coli*, strain 83-1, as described by Davis and Mingioli (12). Specific applications of the procedures are described at appropriate places in the text.

RESULTS

Effect of pH on Inhibition of S. cerevisiae by p-Aminobenzoic Acid—In a preliminary study of factors affecting the assay procedure it was observed that the extent of inhibition of yeast growth by *p*-aminobenzoic acid decreased with increasing pH (Fig. 1). A similar effect of pH on *p*-aminobenzoic acid inhibition of *Aspergillus niger* was noted previously by Cavill and Vincent (4). As the pH was increased, increasing amounts of *p*-aminobenzoic acid were required to give an inhibition of *S. cerevisiae* comparable to that observed at pH 4.5. These observations suggest that a particular form of *p*-aminobenzoic acid, presumably that containing an undissociated carboxyl group, is required for inhibition.

Reversal of p-Aminobenzoic Acid Inhibition by Aromatic Amino Acids—In contrast to the results of Davis (3) with *E. coli*, *p*-hydroxybenzoic acid and shikimic acid were ineffective in reversing *p*-aminobenzoic acid inhibition of *S. cerevisiae* (Table I).[4] Of various substances tested, including sulfanilamide, amino acids, vitamins, purines, and pyrimidines, only the aromatic amino acids were effective in reversing the inhibition. The order of effectiveness of these amino acids was phenylalanine > tyrosine > tryptophan (Table I). Combinations of any two of these amino acids were more effective than the individual amino acids in reversing the inhibition, and a combination of all three amino acids was most effective. With increasing concentration of *p*-aminobenzoic acid, the greater effectiveness of a combination of all three amino acids became more apparent. In fact, a synergistic effect was exhibited by the latter combination. It should be noted, however, that as the concentration of *p*-aminobenzoic acid was raised, increasing amounts of the combination of all three amino acids were required for reversal of the inhibition. At the highest level of *p*-aminobenzoic acid the

* Prepared in part from the doctoral dissertation of Alfred C. Schram, The University of Texas, 1958.

[1] Dilute hydrochloric acid and sodium or ammonium hydroxide were used to adjust the pH of the medium and addenda. When acetic acid was used inhibition of yeast growth was observed.

[2] When substances of limited solubility, *e.g.* tyrosine, were tested, this volume was increased, and a more concentrated basal medium was used to maintain the total volume of medium and addenda at 6 ml.

[3] It was observed many times that sterilization of freshly prepared samples was unnecessary. The relatively low pH of the medium and the short period of incubation precluded interference by other microorganisms.

[4] *p*-Hydroxybenzoic acid and shikimic acid were ineffective also at concentrations of *p*-aminobenzoic acid below the lowest level (300 μg. per 6 ml.) shown in Table I.

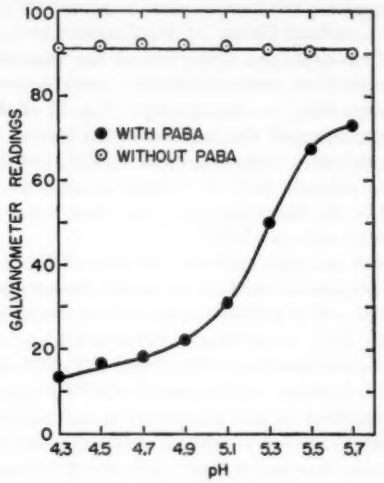

FIG. 1. The effect of pH on inhibition of *S. cerevisiae* by *p*-aminobenzoic acid. Sufficient *p*-aminobenzoic acid was added to the basal medium to give a final concentration of 300 μg. per assay tube, and aliquots of the resulting solution were adjusted (Beckman glass electrode) to the pH values indicated. Other conditions were as described under "Materials and Methods." PABA represents *p*-aminobenzoic acid.

growth rate was not completely restored to normal by the combined aromatic amino acids. Neither shikimic acid nor *p*-hydroxybenzoic acid showed a sparing effect on the aromatic amino acid requirement.

These data suggested that *p*-aminobenzoic acid inhibited, directly or indirectly, the utilization of a common precursor of the aromatic amino acids. The individual amino acids and combinations thereof appeared to exert a sparing effect upon the common precursor. With increasing concentration of *p*-aminobenzoic acid, this sparing effect became less pronounced, suggesting either that *p*-aminobenzoic acid interfered with other enzyme systems or that an additional product derived from the common precursor was required. This postulated product, however, is not *p*-hydroxybenzoic acid, since the latter substance was ineffective when tested in combination with the three aromatic amino acids.

Accumulation of Shikimic Acid in Presence of p-Aminobenzoic Acid—To test the possibility that an aromatic precursor might accumulate in the presence of *p*-aminobenzoic acid, culture filtrates of *S. cerevisiae* were assayed with *E. coli*, strain 83-1. This mutant is blocked between 5-dehydroquinic acid and 5-dehydroshikimic acid, and requires for growth either the latter substance, or shikimic acid, or the five aromatic compounds; phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid (13). Preliminary experiments indicated that the culture filtrates supported slow growth of the mutant on the salts-glucose-citrate medium (medium A) of Davis and Mingioli (14). To increase the sensitivity of the assay, the latter medium was supplemented subsequently with 20 μg. of L-tyrosine and 40 μg. of DL-phenylalanine per ml. (12). The activity of the culture filtrates increased with increasing concentration of *p*-aminobenzoic acid[5, 6] until decreased yeast growth became the limiting factor (Table II). The activity of a filter-sterilized culture filtrate (line 7, Table II) was slightly greater than that of the autoclaved filtrate (line 4, Table II). The latter observation indicated that a small amount of 5-dehydroshikimic acid may have been present in the culture filtrate, since it is known that this substance is largely destroyed by autoclaving at pH 7 (12, 16). However, 5-dehydroshikimic acid was not detectable on bioautographs of the culture filtrate (*cf.* below).

Further information as to the nature of the active material was obtained by bioautography. *E. coli* 83-1 and minimal medium A of Davis and Mingioli (12), supplemented with phenylalanine, tyrosine, and tryptophan, were employed. Use of the supplemented medium enhances the detection of compounds involved in aromatic biosynthesis since the requirements for *p*-aminobenzoic acid and *p*-hydroxybenzoic acid are small com-

TABLE I

Effect of aromatic amino acids, shikimic acid, and p-hydroxybenzoic acid on p-aminobenzoic acid inhibition

p-Aminobenzoic acid	Compound tested*	Galvanometer readings							
		Concentration, μg. per 6 ml.							
		0	10	20	50	100	200	500	1000
μg./6 ml.									
0	None	88							
300	Phenylalanine	21	40	61	81	87			
300	Tyrosine		27	34	54	81			
300	Tryptophan		26	30	33	42			
300	Phenylalanine + tyrosine		51	74	88	87			
300	Phenylalanine + tryptophan		54	77	88	89			
300	Tyrosine + tryptophan		35	45	69	86			
300	Phenylalanine + tyrosine + tryptophan		64	82	88	92			
300	Shikimic acid†		22	21	19	20			
300	*p*-Hydroxybenzoic acid†		23			22			
1000	Phenylalanine	3					29	40	45
1000	Tyrosine						11	16	24
1000	Tryptophan						6	10	15
1000	Phenylalanine + tryosine					38	68	76	81
1000	Phenylalanine + tyrosine + tryptophan					51	84	89	
2000	Phenylalanine + tyrosine + tryptophan					10	22	50	58

* The L-isomers of the amino acids were used. The assay was performed as described under "Materials and Methods."

† Shikimic acid was ineffective also at 200 and 500 μg. levels, and *p*-hydroxybenzoic acid was ineffective at 0.1 and 1.0 μg. levels.

[5] Davis (3) has reported that the minimal inhibitory concentration of *p*-aminobenzoic acid for *E. coli* 83-1 is 10 μg. per ml. on solid medium and even higher in liquid medium. In the present investigation the concentration of *p*-aminobenzoic acid in the *E. coli* 83-1 assays was below 10 μg. per ml., and no evidence of inhibition was noted.

[6] Assay of the yeast culture filtrates by the Bratton-Marshall procedure (15) showed 95 to 100 per cent recovery of *p*-aminobenzoic acid, thus rendering unlikely the possibility that this substance was a precursor of the accumulated compound.

TABLE II

Response of E. coli 83-1 to culture filtrates of inhibited S. cerevisiae

The yeast cultures were set up as described under "Materials and Methods." The second column of figures represents yeast growth in the presence of the concentrations of *p*-aminobenzoic acid listed in the first column. Aliquots of the culture filtrates were assayed with *E. coli* 83-1 as described in the text. The dose response of *E. coli* 83-1 to shikimic acid is included as a control.

p-Aminobenzoic acid	Ml. of culture filtrate			
		0.1	0.3	1.0
	Galvanometer readings			
	S. cerevisiae	*E. coli* 83-1		
μg./6 ml.				
0	89	1	2	2
50	88	1	1	4
100	86	3	8	18
150	69	8	18	38
200	34	6	16	34
250	24	4	12	27
150	69	8*	21*	43*
Shikimic acid				
μg./10 ml.				
0				1
2				17
5				29
10				42
20				52

* Culture filtrate was filter-sterilized; all other samples were autoclaved with the *E. coli* medium.

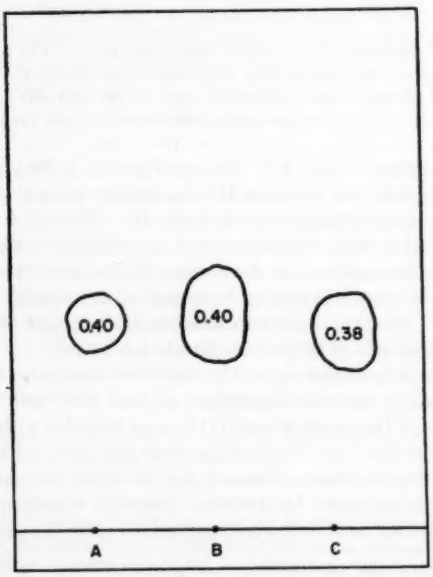

FIG. 2. Tracing of a bioautograph of yeast culture filtrate and shikimic acid. R_F values of zones of growth are given within the zone area: *A*, 0.005 ml. of culture filtrate (Table II, line 7); *B*, mixture of *A* and 0.1 μg. of shikimic acid; *C*, 0.1 μg. of shikimic acid. One-fifth volume of 1 N hydrochloric acid was added to the culture filtrate before chromatography (12, 16).

pared to those for the aromatic amino acids (12). A suitable aliquot of a culture filtrate of *p*-aminobenzoic acid-inhibited yeast, shikimic acid, and a mixture of the two samples were chromatographed on paper with butanol-acetic acid-water as the solvent system (12). A bioautograph (Fig. 2) of the air-dried chromatogram revealed the presence in the culture filtrate of a compound exhibiting chromatographic mobility indistinguishable from that of shikimic acid. 5-Dehydroshikimic acid could not be detected on the bioautographs, even when larger amounts of culture filtrate were employed.[7]

To establish unequivocally the identity of the accumulated compound it appeared desirable to isolate this substance from a culture filtrate of *p*-aminobenzoic acid-inhibited yeast. For this purpose 10.5 l. of medium containing 4.5 gm. of *p*-aminobenzoic acid were inoculated with 18 gm. of Fleischmann bakers' yeast, and the mixture was incubated at 30° for 16 hours. The yeast was removed in a Sharples centrifuge and the effluent (total activity equivalent to 500 mg. of shikimic acid) was filtered through a column prepared from a mixture of 160 gm. of Darco G-60 and 160 gm. of Whatman cellulose powder (coarse grade). The column was washed consecutively with 1.5 l. of water and 1.25 l. of 10 per cent ethanol, and then eluted with 1.25 l. of 50 per cent ethanol. The eluate (total activity, 400 mg. of shikimic acid) was evaporated in a vacuum. The brown syrupy residue was triturated with 10 ml. of warm ethanol, and the ethanol solution was evaporated in a vacuum. The residue was dissolved in 10 ml. of water and the solution was passed through a column prepared from 20 gm. of Dowex 50 × 2 (50-100 mesh, hydrogen phase). The column was washed with 50 ml. of water. Most of the colored material remained in the column. The effluent (total activity, 325 mg. of shikimic acid) was adjusted to pH 8.4 with dilute sodium hydroxide solution and passed through a column prepared from 30 gm. of Dowex 1 × 2 (50 to 100 mesh, chloride phase). The column was washed with 100 ml. of water and then eluted with 100-ml. portions of 0.3 N acetic acid. The second 100-ml. fraction contained most of the activity (total activity, 280 mg. of shikimic acid). This fraction was evaporated in a vacuum, the syrupy residue was dissolved in a hot mixture of 0.8 ml. of ethanol and 3.0 ml. of ethyl acetate, and the solution was stored overnight in a refrigerator. The solid was recrystallized from ethanol-ethyl acetate (1:4) to give 60 mg. of colorless crystals (12 per cent of the original activity). The material isolated had an R_F value in butanol-acetic acid-water identical with that of shikimic acid. The melting point of the isolated material was 185–186° (uncorrected), that of shikimic acid was 186–187° (uncorrected), and the melting point of a mixture of the two samples was 185–187° (uncorrected). The isolated material was characterized further by its x-ray diffraction pattern,[8] which was identical with that of shikimic acid.

Effect of Autoclaving with Acid on Activity of Culture Filtrate—The possibility was considered that a derivative of shikimic acid, *e.g.* compound Z1 or 5-phosphoshikimic acid, not readily detectable by assay with *E. coli* 83-1 under the conditions employed, might be present in the culture filtrate. These two derivatives are converted to shikimic acid by heating in the presence of acid,

[7] On some bioautographs, particularly after prolonged incubation, a large, faint zone of growth (R_F = 0.82) appeared. This spot was due presumably to *p*-aminobenzoic acid which supports slow growth of *E. coli* 83-1 in the presence of the triple aromatic amino acid supplement (12, 13).

[8] The authors are indebted to Dr. S. H. Simonsen for the x-ray diffraction patterns.

and can thus be distinguished from shikimic acid by differential assay (12). The data in Table III show that the activity of the culture filtrate was not altered significantly by autoclaving with 0.1 N hydrochloric acid. Similar results were obtained with a culture filtrate from a heavily inoculated culture prepared as described above. These results indicate that the culture filtrates contained little, if any, compound Z1 or 5-phosphoshikimic acid.[9]

DISCUSSION

It seems reasonable to conclude from the data presented that *p*-aminobenzoic acid, directly or indirectly, inhibits the utilization of a precursor (or precursors) common to the biosynthesis of phenylalanine, tyrosine, and tryptophan. This conclusion is based on the following observations: (*a*) these three amino acids exhibited synergism in reversing *p*-aminobenzoic acid inhibition; (*b*) the individual amino acids were effective, to different extents, in reversing the inhibition (phenylalanine > tyrosine > tryptophan), suggesting that each amino acid exerted a sparing effect upon a common precursor.

The accumulation of shikimic acid in the culture medium in the presence of *p*-aminobenzoic acid suggests that the site of the inhibition is the utilization of endogenous shikimic acid. However, there are several limitations to this suggestion. In the first place, exogenous shikimic acid was ineffective in reversing the inhibition. This lack of activity of shikimic acid may be due to the possibility that externally added shikimic acid is inaccessible to its enzyme within the cell or that the inhibition is noncompetitive in nature. It is also possible that more than one step between shikimic acid and the aromatic amino acids is blocked by *p*-aminobenzoic acid. Although the evidence indicates that neither 5-phosphoshikimic acid nor compound Z1 were present in the culture filtrate, the possibility has not been eliminated that one or both of these compounds did accumulate, but were converted to shikimic acid under the acidic condition of the culture medium. Until additional data are obtained it would seem appropriate to conclude only that the site of inhibition by *p*-aminobenzoic acid is in the sequence of common aromatic precursors between shikimic acid and the aromatic amino acids.

The observation that both *p*-hydroxybenzoic acid and shikimic acid were ineffective in reversing *p*-aminobenzoic acid inhibition of *S. cerevisiae*, whereas *p*-hydroxybenzoic acid, and to a limited extent, shikimic acid, were effective in reversing *p*-aminobenzoic acid inhibition of *E. coli* (3), would appear to indicate that the site of the inhibition is different in these two organisms. However, it should be pointed out that Davis (3) noted that with increasing concentration of *p*-aminobenzoic acid the growth rate of *E. coli* was less completely restored by *p*-hydroxybenzoic acid, and suggested that the residual inhibition was due to interference

TABLE III

Effect of autoclaving with acid on activity of culture filtrate

The yeast culture was set up as described under "Materials and Methods;" pH, 4.5; *p*-aminobenzoic acid concentration, 300 μg. per 6 ml. One-half the culture filtrate was autoclaved for 30 minutes with an equal volume of 0.2 N hydrochloric acid and the hydrolysate was neutralized with dilute sodium hydroxide. Equivalent amounts of the treated and untreated samples were assayed with *E. coli* 83-1 as described in the text.

Treatment	Ml. of culture filtrate		
	0.1	0.3	1.0
	Galvanometer readings		
None	4	11	31
Acid-heat	3	10	28

of *p*-aminobenzoic acid with other enzyme systems. It may be that *p*-hydroxybenzoic acid does not play a metabolic role in *S. cerevisiae*, and that the enzyme system (or systems) in *E. coli* to which Davis alluded has a function similar to the enzyme system in *S. cerevisiae* which is inhibited by *p*-aminobenzoic acid. It should be noted also that the pH of the *E. coli* medium was 7, whereas the present experiments with *S. cerevisiae* were performed at pH 4.5. The significance, if any, of this difference remains to be studied. As mentioned earlier in this paper, the extent of inhibition of *S. cerevisiae* by *p*-aminobenzoic acid decreased as the pH was increased above the latter value.

SUMMARY

Growth of *Saccharomyces cerevisiae* is inhibited by high concentrations of *p*-aminobenzoic acid. The minimal inhibitory concentration was approximately 25 μg. per ml. The extent of inhibitition decreased as the pH was increased above 4.5.

Of various substances tested, only the aromatic amino acids were effective in reversing the inhibition. The order of effectiveness of these amino acids was phenylalanine > tyrosine > tryptophan. Combinations of any two of these amino acids were more effective than the individual amino acids and a combination of all three amino acids was most effective.

An aromatic precursor accumulated in the culture medium in the presence of *p*-aminobenzoic acid. This precursor was isolated and identified as shikimic acid.

It is suggested that *p*-aminobenzoic acid inhibits, directly or indirectly, the utilization of shikimic acid or a subsequent common precursor of the aromatic amino acids.

Acknowledgment—We wish to thank Janet G. Reed for excellent technical assistance.

REFERENCES

1. YEOMANS, A., SNYDER, J. C., MURRAY, E. S., ZARAFONETIS, C. J. D., AND ECKE, R. S., *J. Am. Med. Assoc.*, **126**, 349 (1944).
2. SEVAG, M. G., *Advances in Enzymol.*, **6**, 33 (1946).
3. DAVIS, B. D., *J. Exptl. Med.*, **94**, 243 (1951).
4. CAVILL, G. W. K., AND VINCENT, J. M., *J. Soc. Chem. Ind. London*, **67**, 25 (1948).
5. LAVOR, E. M., AND FERGUSON, F. F., *J. Elisha Mitchell Sci. Soc.*, **58**, 166 (1942); *Chem. Abstr.*, **37**, 2083 (1943).
6. MANGENOT, G., AND CARPENTIER, S., *Compt. rend. soc. biol.*, **135**, 1053, 1152 (1941).
7. SNYDER, J. C., AND DAVIS B. D., *Federation Proc.*, **10**, 419 (1951).

[9] After completion of this investigation a sample of compound Z1 was obtained through the kindness of Drs. D. B. Sprinson and B. D. Davis. Dr. P. C. Shah in this laboratory has observed that compound Z1 is ineffective in reversing *p*-aminobenzoic acid inhibition of *S. cerevisiae* when tested alone or in combination with the three aromatic amino acids.

8. Takemori, N., and Kitaoka, M., *Science*, **116**, 710 (1952).
9. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).
10. Snell, E. E., Eakin, R. E., and Williams, R. J., *University of Texas, Publication Number 4137*, 1941.
11. Williams, R. J., McAlister, E. D., and Roehm, R. R., *J. Biol. Chem.*, **83**, 315 (1929).
12. Davis, B. D., and Mingioli, E. S., *J. Bacteriol.*, **66**, 129 (1953).
13. Davis, B. D., *J. Bacteriol.*, **64**, 729 (1952).
14. Davis, B. D., and Mingioli, E. S., *J. Bacteriol.*, **60**, 17 (1950).
15. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537 (1939).
16. Davis, B. D., *J. Biol. Chem.*, **191**, 315 (1951).

Enzymatic Sulfurylation of Tyrosine Derivatives*

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A variety of nonphysiological phenols (1–4), as well as phenolic (5–7) and nonphenolic (6, 8) steroids, have been shown to be capable of undergoing sulfurylation in the presence of a suitable 3′-phosphoadenosine-5′-phosphosulfate-generating system and sulfate transferring enzyme from liver. Attempts to sulfurylate the phenolic amino acid, tyrosine, however, were unsuccessful (9). This finding was particularly puzzling in view of the fact that tyrosine-*O*-sulfate is excreted in appreciable quantities in human urine (10), and that in at least one protein tyrosine occurs in a sulfurylated form (11). It occurred to us that derivatives of tyrosine, rather than free tyrosine, might be the forms which undergo sulfurylation. Experiments demonstrating that this is indeed the case are reported in the present communication.

EXPERIMENTAL

Enzyme Preparation—The livers of normal, male rats of the Carworth strain were homogenized in 9 volumes of 0.25 M sucrose containing 10^{-3} M ethylenediaminetetraacetate in a Servall Omni-Mixer for 30 seconds at 0°. The homogenate was centrifuged at 5,000 × *g* for 10 minutes, and the supernatant fluid recentrifuged at 100,000 × *g* for 20 minutes. GSH, 0.1 M, was added to the second supernatant fluid to a final concentration of 0.01 M (fraction S_2).

Assay Procedures—The enzyme activity was based upon the rate of appearance of free *p*-nitrophenol, which was measured by the optical density change at 400 mμ in a Beckman model DU spectrophotometer, essentially according to the method of Gregory and Lipmann (4). In the complete system, each cuvette contained in 3.2 ml., 15 μmoles of *p*-nitrophenyl sulfate, 450 μmoles of tris(hydroxymethyl)aminomethane or glycine buffer, 15 μmoles of sulfate acceptor, 30 μmoles of GSH, and 0.3 ml. of coenzyme solution. The reaction was started by the addition of the enzyme solution by means of the adder-mixer invented by Boyer (12). Water at 30° was circulated around the cell compartment. Plots of optical density against time were linear for the first few minutes, and the initial slope was taken as the initial velocity of the reaction. The rate was proportional to enzyme concentration within the limits employed.

Protein was determined by the method of Lowry *et al.* (13). A bovine albumin preparation was employed as a standard.

Materials—*p*-Nitrophenyl sulfate was the potassium salt (Sigma Chemical Company). The coenzyme solution was prepared from a crude barium adenosine diphosphate fraction (Schwarz Laboratories, Inc.) and was about 0.007 M in adenine compounds as determined spectrophotometrically.

* This work was supported by a research grant (A-875) from the United States Public Health Service.

RESULTS

Acceptor Specificity—A variety of tyrosine derivatives was tested for acceptor activity as listed in Table I. The rate with *m*-aminophenol as acceptor is set at 100 at each pH for purposes of comparison. Of the tyrosine derivatives tested only those with a free amino group and without a free carboxyl group exhibited significant activity.

pH versus Activity—It is apparent from the pH versus activity curves (Fig. 1) that the pH optimum with L-tyrosine ethyl ester as acceptor was markedly higher than that with *m*-aminophenol as acceptor. L-Tyrosine methyl ester, tyrosine amide, and especially tyramine were also found to elicit greater activity at pH 9.3 than at pH 7.8 (Table I).

Ammonium Sulfate Fractionation—A 4-fold purification with approximately two-thirds recovery could be achieved by precipitation of the enzyme from the S_2 fraction with 25 to 50 per cent saturated ammonium sulfate (AS-1 fraction), as shown in Table II. The ammonium sulfate was added as a saturated solution (4°) adjusted to pH 7.6 with concentrated ammonia. A further purification of about 50 per cent with a poorer recovery was possible by reprecipitating from the AS-1 fraction (containing 4 to 5 mg. of protein per ml.) with 30 to 40 per cent saturated ammonium sulfate (AS-2 fraction). The precipitates were dissolved in 0.05 M tris(hydroxymethyl)aminomethane, pH 7.8, containing 0.01 M GSH.

It can be seen from Table II that there was no significant separation of *m*-aminophenol and L-tyrosine ethyl ester sulfurylating activities by this procedure.

Coenzyme Requirement—A crude adenosine diphosphate preparation proved to be a source of coenzyme activity for sulfate transfer (Fig. 2), as also reported by Gregory and Lipmann (4), who have identified the active compound as 3′,5′-diphosphoadenosine. From the extent of reaction in the absence of added acceptor (*Curve C*) and the equilibrium constant for the transfer of sulfate from *p*-nitrophenyl sulfate to 3′,5′-diphosphoadenosine (4),[1] it can be calculated that the 3′,5′-diphosphoadenosine concentration of the incubation solution was 4 to 5 mμmoles per ml., and that this compound, therefore, represented 0.5 to 1 per cent of the adenine compounds present in the coenzyme solution.

Repeated precipitation of the enzyme with ammonium sulfate caused a progressive reduction in the ratio of the activity in the absence and presence of added coenzyme, as can be seen from

[1] The difference between the pH at which the equilibrium constant was obtained (7.8) and the pH of the present measurements (8.3) can be ignored, since, at the ratio of *p*-nitrophenyl sulfate to *p*-nitrophenol present (1000:1), essentially all the 3′,5′-diphosphoadenosine is converted to 3′-phosphoadenosine-5′-phosphosulfate at either pH.

TABLE I

Comparison of various compounds as sulfate acceptors

Assay conditions as described in text with tris(hydroxymethyl)-aminomethane buffer, pH 7.8, or glycine buffer, pH 9.3. The enzyme was from the S_2 fraction. Relative activities were calculated from the change in optical density between 1 and 15 minutes after addition of the enzyme, minus the corresponding change in the absence of added acceptor.

Compounds	Relative degree of sulfurylation	
	pH 7.8	*pH 9.3*
m-Aminophenol*	100	100
L-Tyrosine ethyl ester†	20	52
L-Tyrosine methyl ester‡	21	39
N-acetyl-L-tyrosine ethyl ester†	0	1
Carbobenzoxy-L-tyrosine methyl ester‡	0	3
L-Tyrosine§	1	1
L-Arginyl-L-tyrosine‡	0	—[a]
L-Seryl-L-tyrosine‡	0	—
Carbobenzoxy-L-seryl-L-tyrosine‡	0	—
L-Tyrosine amide¶	0	8
L-Tyrosine hydroxamide¶	0	—
Tyramine¶	7	68
N-acetyl-hydroxy-L-proline‡	4	0
Hydroxy-L-proline¶	—	0
L-Phenylalanine ethyl ester¶	—	0
Acetyl-ser.tyr.ser.met.glu-NH_2‖	—	0
L-Tyrosyl glycine‖	0	3

* Eastman Kodak Company, treated with charcoal and recrystallized twice from hot water.

† Gift of Dr. C. J. Martin.

‡ Gift of Dr. K. Hofmann.

§ Eimer and Amend.

¶ Mann Research Laboratories, Inc.

‖ Gift of Dr. Sofia Simmonds.

[a] Not determined.

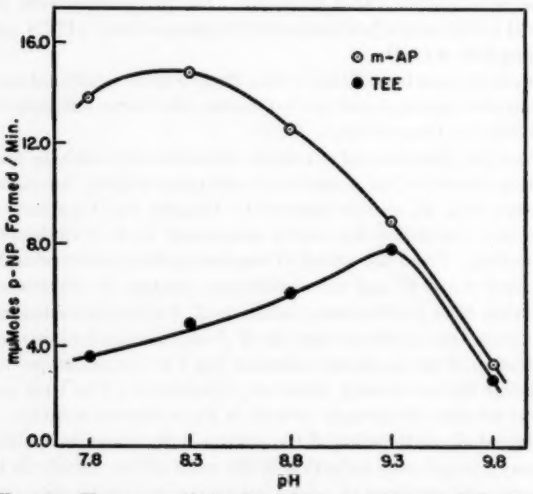

FIG. 1. pH *versus* activity for sulfate transfer. Tris(hydroxymethyl)aminomethane buffer was used in the range 7.8 to 8.8 and glycine buffer in the range 8.8 to 9.8. The amount of *p*-NP (*p*-nitrophenol) formed was calculated by means of the extinction coefficients appropriate to each pH and was corrected at the higher pH values for a slight inhibition by glycine (about 10 per cent). Either *m*-AP (*m*-aminophenol) or TEE (L-tyrosine ethyl ester) was present as sulfate acceptor as indicated. An aliquot of an S_2 fraction containing 1.6 mg. of protein was the source of the enzyme in all cases.

TABLE II

Ammonium sulfate fractionation of sulfate transfer activity

Data are given with both *m*-AP (*m*-aminophenol) as acceptor in presence of tris(hydroxymethyl)aminomethane buffer, pH 8.3, and TEE (L-tyrosine ethyl ester) as acceptor in presence of glycine buffer, pH 9.3. Other conditions as described in text.

Fraction*	Specific activity		Activity ratio: $\frac{\text{without added coenzyme}}{\text{with added coenzyme}}$	
	with *m*-AP	with TEE	with *m*-AP	with TEE
	(*units/mg. protein*)†			
S_2	7.46	3.64	.76	.19
AS-1	30.4	15.8	.22	.06
AS-2	44.4	24.2	.15	.00

* Preparation of these fractions described in text.

† Unit defined as amount of activity catalyzing transfer of 1 mμmole of sulfate per minute.

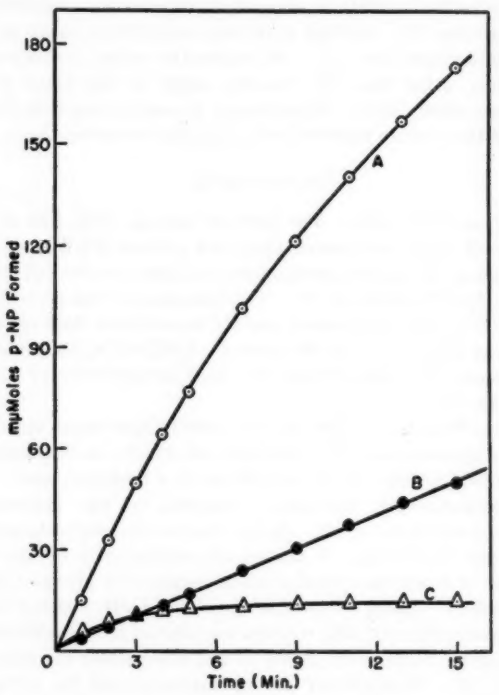

FIG. 2. Time course of sulfate transfer. In *Curve A* the complete system was present as described in the text, with *m*-aminophenol as sulfate acceptor and tris(hydroxymethyl)aminomethane buffer, pH 8.3. In *Curves B* and *C* added coenzyme and acceptor, respectively, were omitted. An aliquot of an AS-1 fraction containing 0.47 mg. of protein was the source of the enzyme in all cases. *p*-NP refers to *p*-nitrophenol.

the last two columns of Table II, leading to an almost absolute requirement for added coenzyme after the second precipitation. Treatment of the S_2 fraction with Norit or Dowex 1-chloride also accentuated the coenzyme requirement.

No liberation of *p*-nitrophenol was observed when added acceptor and coenzyme were both absent, thus indicating that arylsulfatase activity was not present in the enzyme preparations.

Closely similar results in regard to pH characteristics, added

coenzyme requirement and substrate specificity were obtained with a specimen of normal human liver.[2]

DISCUSSION

The observation of Bettelheim (11) that the hydroxyl groups of the tyrosine residues in fibrinogin are esterified with sulfate, and of Tallan *et al.* (10) that tyrosine-*O*-sulfate is a normal excretory product established the fact that tyrosine or some derivative thereof undergoes sulfurylation *in vivo*. The inability of tyrosine itself to be sulfurylated apparently excludes the possibility that tyrosine-*O*-sulfate is merely an artifact of a detoxification type of reaction. It is interesting to note also that tyrosine-*O*-sulfate is not a substrate for a mammalian liver arylsulfatase (14).

The present observations, that only carboxyl-substituted or carboxyl-lacking derivatives of tyrosine possessing a free amino group undergo sulfurylation in the sulfate esterifying system of liver, suggest that an analogous type of derivative is the naturally occurring sulfate acceptor, possibly an *N*-terminal tyrosine peptide in which the free carboxyl group is sufficiently remote or lacking entirely. The quantities of tyrosine-*O*-sulfate found in urine by Tallan *et al.* (10) represent a sizable fraction of the total tyrosine excreted. If it arises from the breakdown of peptide-bound material, it would suggest that the total pool of sulfurylated protein is larger than heretofore supposed, or that it undergoes a rapid turnover. It is entirely possible that a number of proteins occur naturally in a sulfurylated form, since in the usual procedures for the analysis of the composition of proteins the sulfate groups would be rapidly hydrolyzed, and sulfate is usually not sought among the hydrolysis products.[3]

It is not possible, on the basis of the present evidence, to decide whether sulfurylation of the tyrosine derivatives is catalyzed by the same enzyme as that involved in the case of the simple phenols. Precipitation of the enzyme with ammonium sulfate, as described here, does not lead to a separation of activity, and a common coenzyme requirement exists in both cases. On the other hand, in each enzyme fraction studied the L-tyrosine ethyl ester sulfurylating activity was appreciably more dependent upon added coenzyme than the *m*-aminophenol sulfurylating activity, and there was a marked difference in the pH optima for the two acceptors. It is probable, however, that the higher pH optimum in the case of the tyrosine derivatives reflects an influence of pH on both the enzyme and the aliphatic amino group of the side chain, particularly in view of the pronounced effect of substitution on the amino group on the ability of the methyl and ethyl esters of L-tyrosine to act as sulfate acceptors.

SUMMARY

A number of tyrosine derivatives have been tested as sulfate acceptors in a liver sulfate transfer system. Only those compounds in which the carboxyl group was absent or substituted and in which the amino group was unsubstituted underwent sulfurylation. The implications of these findings regarding the mechanism of formation of sulfurylated tyrosine compounds *in vivo* is discussed.

A markedly higher pH optimum was observed for sulfurylation of tyrosine derivatives than for sulfurylation of simple phenols. It is considered probable that this difference reflects a requirement for an uncharged amino group on the side chain in the former class of compounds.

Ammonium sulfate fractionation did not lead to a separation of *m*-aminophenol and tyrosine ethyl ester sulfurylating activities. However, there was a notable difference between these activities in their dependence upon added coenzyme.

REFERENCES

1. De Meio, R. H., and Tkacz, L., *J. Biol. Chem.*, **195**, 175 (1952).
2. Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **198**, 195 (1952).
3. Sato, S., Suzuki, T., and Fukuyama, T., *J. Biochem.*, **43**, 421 (1956).
4. Gregory, J. D., and Lipmann, F., *J. Biol. Chem.*, **229**, 1081 (1957).
5. Segal, H. L., *J. Biol. Chem.*, **213**, 161 (1955).
6. Roy, A. B., *Biochem. J.*, **63**, 294 (1956).
7. De Meio, R. H., Lewycka, C., Wizerkaniuk, M., and Salciunas, O., *Biochem. J.*, **68**, 1 (1958).
8. De Meio, R. H., and Lewycka, C., *Endocrinology*, **56**, 489 (1955).
9. Segal, H. L., *Federation Proc.*, **16**, 245 (1957).
10. Tallan, H. H., Bella, S. T., Stein, W. H., and Moore, S., *J. Biol. Chem.*, **217**, 703 (1955).
11. Bettelheim, F. R., *J. Am. Chem. Soc.*, **76**, 2838 (1954).
12. Boyer, P. D., and Segal, H. L., in W. D. McElroy and B. Glass (Editors), *The mechanism of enzyme action*, Johns Hopkins Press, Baltimore, 1954, p. 523.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
14. Dodgson, K. S., and Wynn, C. H., *Biochem. J.*, **68**, 387 (1958).
15. Dziewiatkowski, D. D., and Di Ferrante, N., *J. Biol. Chem.*, **227**, 347 (1957).

[2] Obtained 3 to 4 hours post-mortem through the courtesy of the Pathology Department, Presbyterian Hospital, Pittsburgh.

[3] In this connection, it appears that a portion of the sulfur found to be associated with serum proteins by Dziewiatkowski and Di Ferrante (15) may be in the form of peptide-linked sulfate.

The Effect of Riboflavin Deficiency upon the Metabolism of Tryptophan by Liver and Kidney Tissue*

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Riboflavin-deficient rats have been observed to excrete considerably more kynurenic acid than normal rats after the ingestion of L-tryptophan, but only slightly more kynurenine and xanthurenic acid (1). Increased excretion of anthranilic acid has also been noted (2, 3). Although the administration of tryptophan by injection also produces an increased excretion of xanthurenic acid in riboflavin deficiency, the injection of kynurenine is reported to yield somewhat less in the deficient than in the normal animal (4).

Riboflavin is a component of the prosthetic groups of D- and L-amino acid oxidase. Its lack might therefore be assumed to alter the production of β-3-indolepyruvic acid, and through such alteration to influence the production and output of other tryptophan metabolites. This hypothesis has been tested by determining the metabolite production after the incubation of liver and kidney slices from riboflavin-deficient and normal animals with L-, DL-, and D-tryptophan, L- and D-kynurenine, and indolepyruvic acid. The study has required the preliminary development of a suitable method of measuring indolepyruvic acid quantitatively and the adaptation of other analytical procedures to the conditions employed. The purpose of this paper is to describe the results obtained by this approach.

EXPERIMENTAL

Materials—The DL-tryptophan used in these studies was a recrystallized synthetic product.[1] The D- and L-tryptophan were prepared through resolution of the brucine salt of acetyl-DL-tryptophan[1] by the method of Shabica and Tishler (5). For the D-tryptophan, the $[\alpha]_D^{25}$, at a concentration of 0.5 gm./100 ml. of solution in water, was +31.1°, for the L-form it was −30.8°. The D- and L-kynurenine, used as the sulfate monohydrates, were from lots available in the laboratory (6). The $[\alpha]_D^{25}$, at a concentration of 1.0 gm./100 ml. of solution in water, was − 9.5° for the former, + 9.6° for the latter. The indolepyruvic acid was synthesized as outlined elsewhere (7) by condensing indole-3-aldehyde, prepared as directed by Boyd and Robson (8), with hippuric acid and hydrolyzing the azlactone with alkali. The purified product ultimately obtained was light tan in color, m.p. 211–212°. Its *p*-nitrophenylhydrazone melted at 153°, in good agreement with the literature (7, 9).

Tissue Slices—The riboflavin-deficient and the normal rats were males of the Sprague-Dawley strain. They had been housed as weanlings in individual cages and had been fed diets which contained "vitamin-free" casein (Nutritional Biochemicals) 18.0, DL-methionine 0.2, sucrose 50.0, Cellu flour 2.0, salt mixture (Jones and Foster (10)) 4.0, Crisco 23.0, corn oil 2.0, vitamin A and D concentrate[2] 0.08, choline chloride 0.2, liver concentrate[3] 0.4, and inositol 0.1 per cent. Each kg. of diet was fortified normally with a vitamin supplement consisting of thiamine hydrochloride 5 mg., riboflavin 10 mg., pyridoxine hydrochloride 5 mg., nicotinic acid 5 mg., calcium pantothenate 25 mg., *p*-aminobenzoic acid 300 mg., α-tocopherol acetate 25 mg., 2-methyl-1,4-naphthoquinone 2 mg., biotin 100 μg., folic acid 100 μg., and vitamin B_{12} 15 μg. After 3 weeks, when the individual weights approximated 145 to 150 gm., riboflavin was removed from the diet of half of the animals. In 13 to 17 weeks thereafter bilateral and symmetrical alopecia, conjunctivitis and severe ophthalmia, partial paralysis of the legs, and marked loss in weight were observed as signs of the deficiency. At this stage, the rat was killed by a blow on the head and the liver and kidneys were removed and placed in Petri dishes containing cold freshly mixed Krebs-Henseleit solution (11). Control tissues were similarly obtained from rats fed the normal diet throughout. Tissue slices, 0.3 to 1 mm. thick, were prepared with a Stadie-Riggs slicer, washed, and kept in cold Krebs-Henseleit solution in Petri dishes surrounded with crushed ice before use.

Incubation—Washed tissue slices, 400 mg. wet weight, were incubated in Warburg vessels for 3 hours at 37° in an atmosphere of O_2 in a medium consisting of 2 ml. of Krebs-Henseleit buffer, pH 7.4 (11), or 2 ml. of sodium pyrophosphate buffer, pH 8.2 (12), plus 1 ml. of buffered substrate. Per ml., the buffered substrate contained 1 mg. of tryptophan (D-, L-, or DL-), kynurenine sulfate (D- or L-), or indolepyruvic acid. After the incubation, the medium was decanted from the tissue and aliquots were used for analysis.

Methods of Analysis—Depending upon the substrate employed and the response to paper chromatography, the media were analyzed for all or several of the following: tryptophan, indole-

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[1] We are indebted to The Dow Chemical Company of Midland, Michigan, for the DL-tryptophan and the acetyl-DL-tryptophan supplied.

[2] Oleum percomorphum, Mead Johnson and Company, containing not less than 60,000 vitamin A units and 8500 vitamin D units, U.S.P., per gm.

[3] Liver concentrate, N. F., kindly supplied by The Wilson Laboratories, through the courtesy of Dr. S. W. Hier.

pyruvic acid, kynurenine, anthranilic acid, kynurenic acid, and xanthurenic acid.

Indolepyruvic acid gives a color when spotted on paper and sprayed with Ehrlich's reagent (0.2 per cent solution of *p*-dimethylaminobenzaldehyde in 2 N HCl in 80 per cent ethanol); with Salkowski's reagent (0.01 M $FeCl_3$ in 35 per cent $HClO_4$); or with diazotized sulfanilic acid (13).

The paper chromatographic technique recommended by Stowe and Thimann (13) for the separation of indole derivatives was first tried without success. When Whatman No. 1 filter paper strips were spotted with 50 to 800 μg. of indolepyruvic acid and developed with isopropanol, water, and ammonia, in an 8:1:1 ratio, no authentic indolepyruvic acid spot could be detected. Marked streaking, indicative of extensive destruction, was observed. Subsequently Bentley *et al.* (14) recorded a similar experience, but reported that the destruction could be avoided by the use of an acidic solvent. With the solvent of Mason and Berg (15), made acid by the addition of 2 ml. of glacial acetic acid per 100 ml. (16), definite indolepyruvic acid spots were detected. However, subsequent assays by the procedure outlined below showed that, even when the solvent was thus modified, marked degradation (60 to 80 per cent) occurred.

Application of the colorimetric method of Sealock (17) for α-keto acids to the estimation of indolepyruvic acid, whether in eluates or in pure solution, produced an interfering turbidity. This could be avoided by extracting the reaction mixture with ether, evaporating the solvent, and partitioning the residue between equal volumes of ammonium hydroxide solution and chloroform, as directed by Cavallini and Frontali (18). The phenylhydrazones of the keto acids are soluble in the former, the neutral phenylhydrazones and phenylhydrazine in the latter. When 150 to 900 μg. of indolepyruvic acid were added to the media, application of this procedure accounted for 92 to 98 per cent. Deproteinization with trichloroacetic acid or with tungstic acid before the analysis reduced the recovery to 73 to 81 per cent. When media to which indolepyruvic acid or tryptophan had been added before incubation were tested for extraneous α-keto acids by chromatographing the dinitrophenylhydrazone fraction, none could be detected.

The following method of estimation was therefore adopted. To 1 ml. of 0.2 per cent 2,4-dinitrophenylhydrazine in 0.2 N HCl solution was added 0.5 to 1.0 ml. of the incubated medium. After 1 hour at room temperature the mixture was chilled in an ice bath and extracted with successive 2-ml. portions of ice-cold ether until none of the yellow color remained. The combined ether fractions were evaporated to dryness at room temperature under reduced pressure. The dried residue was shaken well with 4 ml. of freshly prepared 1 N ammonium hydroxide solution. An equal volume of chloroform was added, the mixture again shaken thoroughly and centrifuged to obtain a clear ammonium hydroxide layer.

Two ml. of the ammonium hydroxide layer were pipetted into a 10-ml. glass-stoppered graduated cylinder. One ml. of 2 N sodium hydroxide solution was added to develop the red color. After 5 minutes the mixture was diluted to 8 ml. with water, mixed again, and allowed to stand for an additional 7 minutes. The color was then measured at 510 mμ in the Coleman spectrophotometer. Indolepyruvic acid values were computed from a standard curve prepared from similarly treated solutions of pure indolepyruvic acids. The test is sensitive to 10 μg. of indolepyruvic acid in 8 ml.

Tryptophan was estimated by the diazotization technique previously described (19, 20). Kynurenine, anthranilic acid, and indolepyruvic acid also respond. Upon the addition of the sodium nitrite solution to an admixture, indolepyruvic acid gives a purplish color which persists until the final colorimetric assay. On Whatman No. 1 filter paper strips the acidified Mason and Berg solvent (15, 16) effectively separated the anthranilic and indolepyruvic acids chromatographically from the tryptophan and the kynurenine. The two former substances migrated much more rapidly (R_F approximately 0.94) than the two latter (R_F approximately 0.44 and 0.42). However, relatively large amounts of tryptophan and kynurenine produced considerable overlapping. The kynurenine could be determined without interference by the tryptophan because it required much less drastic conditions than the latter for diazotization. Moderate amounts of kynurenine could readily be destroyed by incubating the diazotizate at 37° for 1 hour (19, 21), but when large amounts were present, the destruction was not complete. Heating with sodium hydroxide and sodium carbonate at 100° for 3 hours (22) destroyed the kynurenine completely, but also produced a partial loss of the tryptophan. Since the use of large amounts of medium seemed desirable, Tabone's solvent (5 ml. of concentrated H_2SO_4 per l. of 80 per cent ethanol) was tried (21). This produced a wide enough separation of the tryptophan (R_F 0.56) and the kynurenine (R_F 0.46) to eliminate the overlapping completely in most instances, or to decrease it to the point where the residual kynurenine could readily be destroyed by incubation after diazotization. Interference by indoleacetic acid, which could have been produced from the indolepyruvic acid, was ruled out because indoleacetic acid had a relative R_F value of 0.8 when chromatographed with Tabone's solvent.

In determining tryptophan, therefore, 0.2 ml. of medium was spotted on Whatman No. 1 filter paper sheets, along with control spots of 0.07 ml. of L- and D-tryptophan solutions (1 mg. per ml.) Cylinders prepared from the sheets were allowed to stand in air tight vessels for 12 to 14 hours to allow the Tabone solvent to ascend. The spots produced from the L- and D-tryptophan were located by spraying the dried paper with Ehrlich's reagent. The corresponding unsprayed areas were cut out and clipped into narrow strips for elution with successive 2-ml. portions of freshly prepared 4 per cent trichloroacetic acid solution, followed by centrifugation, until 10 ml. of eluate and washings had been obtained. To these 10 ml., 1 ml. of 0.1 per cent sodium nitrite solution was added. After 3 minutes, the mixture was incubated for 1 hour to destroy any contaminating kynurenine. One ml. of 1 per cent sodium nitrite was then added, followed in 30 minutes by 0.5 ml. of 8 per cent ammonium sulfamate solution, and in 3 more minutes by 1 ml. of 0.1 per cent *N*-1-naphthylethylenediamine hydrochloride solution. The purple color was assayed 30 minutes later at 560 mμ in the Coleman spectrophotometer. The tryptophan content was calculated from a standard calibration curve. The method responded well to 5 μg. of tryptophan in 13.5 ml. of solution.

Kynurenine was estimated by a procedure similar to that described for tryptophan, using 0.2 ml. of medium, but in this instance irrigating for 10 to 12 hours with the acidified solvent of Mason and Berg (15, 16). The control spots produced with 0.05 ml. of L- and D-kynurenine sulfate solutions (1 mg. per ml.) and the kynurenine spots produced from the incubated media were located by their fluorescence under ultraviolet light. The 10 ml. of eluate obtained from each of the kynurenine spots were

mixed with 1 ml. of 0.1 per cent sodium nitrite solution. After 3 minutes, excess nitrite was destroyed by the addition of 1 ml. of 4 per cent ammonium sulfamate. Three minutes later 1 ml. of 0.1 per cent *N*-1-naphthylethylenediamine hydrochloride solution was added to develop the color. Readings were made at 560 mμ. Kynurenine values were calculated from the standard kynurenine calibration curve. The sensitivity was approximately the same as for tryptophan.

Anthranilic acid also produces spots on paper which can be located by their fluorescence in ultraviolet light (15). The same chromatograms were used for estimating anthranilic acid as had been used for kynurenine. Conditions for the elution of the anthranilic acid spots, the diazotization, and the color development were the same. The amount of anthranilic acid present in the medium was calculated from standard calibration curves prepared with anthranilic acid. As little as 4 μg. in 13 ml. was readily measurable.

Neither the solvent of Tabone (22) nor the acidified solvent of Mason and Berg (15, 16) will effectively separate indolepyruvic acid chromatographically from anthranilic acid. When present in appreciable amount, indolepyruvic acid increases the intensity of color developed in the assay method for anthranilic acid. Eluates of sections of control chromatograms of mixtures of indolepyruvic acid and anthranilic acid prepared with the acidified Mason and Berg solvent, which permits marked destruction of the indolepyruvic acid during the irrigation, failed to show the interference noted in direct tests of the mixtures for anthranilic acid. Evidently the destruction of the indolepyruvic acid is complete enough to avoid appreciable interference.

Xanthurenic acid was estimated by deproteinizing 1 to 2 ml. of the medium with 4 per cent trichloroacetic acid solution, and by adding 0.1 ml. of 0.425 per cent $FeNH_4(SO_4)_2$ and 2 ml. of 1 N sodium bicarbonate solution, essentially as directed by Glazer *et al.* (23). After half an hour the green color was measured at 620 mμ in the Coleman spectrophotometer and the concentration was computed from comparisons with a calibration curve prepared with standard solutions of xanthurenic acid. The method is sensitive to 15 μg. in 8 ml. Neither tryptophan nor any of the other metabolites studied respond, nor do media incubated without added substrate.

Kynurenic acid in 20 per cent trichloroacetic acid solution absorbs strongly at 312 mμ (20), xanthurenic acid less strongly. Indolepyruvic acid interferes, but can be eliminated by the use of paper chromatograms developed with the acidified solvent of Mason and Berg, with which it shows an R_F value of 0.94 *versus* 0.58 and 0.56 for kynurenic acid and xanthurenic acid, respectively.

The same chromatographic technique was applied as for kynurenine and anthranilic acid, but 0.5 ml. of medium was applied to the paper and elution of the fluorescing spot was effected with a 20 per cent solution of trichloroacetic acid instead of 4 per cent. Kynurenic acid is relatively insoluble in the latter. Optical densities of the eluate were measured at 312 mμ in the Beckman DU spectrophotometer and the kynurenic acid content was calculated from a curve prepared with standard kynurenic acid solutions. Corrections based on the calculated interference of the xanthurenic acid present in the media were applied. The test was sensitive to 2 μg. of kynurenic acid in 10 ml. of solution.

RESULTS AND DISCUSSION

Tables I and II present summaries of the data obtained when liver and kidney slices from normal (Table I) and riboflavin-deficient (Table II) rats were incubated with tryptophan and with kynurenine. More kynurenine accumulated when DL- or L-tryptophan was incubated with liver slices from the riboflavin-deficient rat than when normal liver was employed. The same was true of kynurenic acid and anthranilic acid, whether the substrate was DL- or L-tryptophan or L-kynurenine. Too little xanthurenic acid was produced by normal liver or kidney to estimate, but fairly large amounts accumulated when DL- or L-tryptophan or L-kynurenine was incubated with these tissues from the riboflavin-deficient animal. Kidney tissue produced kynurenic acid from both D- and L-kynurenine, but not from tryptophan. The amount which accumulated upon incubation with L-kynurenine was much larger with kidney from the riboflavin-deficient animal. Neither type of liver slice produced

TABLE I

Production of metabolites upon incubation of tryptophan and kynurenine with liver and kidney slices from normal rats

The following abbreviations are used in this and the succeeding tables: tryptophan, T; kynurenine, K; indolepyruvic acid, IPA; kynurenic acid, KA; anthranilic acid, AA; xanthurenic acid, XA.

No. of rats	Substrate*	Liver slice tests†					Kidney slice tests†		
		Substrate recovered	Metabolites found				Substrate recovered	Metabolites found	
			IPA	K	KA	AA		IPA	KA
		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
5	D-T	477 ± 62‡	40 ± 5	0	24 ± 4	0	102 ± 19	730 ± 42	0
5	DL-T	298 ± 29	48 ± 9	77 ± 12	37 ± 8	41 ± 10	180 ± 17	552 ± 31	0
5	L-T	349 ± 28	72 ± 15	161 ± 15	66 ± 8	65 ± 10	480 ± 17	164 ± 41	0
5	D-K	402 ± 21	0		0	0	352 ± 41	0	96 ± 15
5	L-K	336 ± 24	0		42 ± 5	96 ± 9	168 ± 10	0	174 ± 17

* In each test 1000 μg. of substrate in 3 ml. of Krebs-Henseleit buffer, pH 7.4, were incubated with 400 mg. of tissue slices for 3 hours in an atmosphere of O_2. The K used as substrate was the hydrated sulfate; the 1000 μg. were equivalent to 643 μg. of K. Recoveries and assays are recorded as K.

† No XA production was observed by either liver or kidney slices from the normal, well fed rat, with any of the substrates. Kidney slices failed to produce K from T, or to yield AA from any of the substrates.

‡ ± = Standard deviation of the mean.

TABLE II

Production of metabolites upon incubation of tryptophan and kynurenine with liver and kidney slices from riboflavin-deficient rats

No. of rats	Substrate*	Liver slice tests†					Kidney slice tests†			
		Substrate recovered	Metabolites found				Substrate recovered	Metabolites found		
			K	KA	XA	AA		IPA	KA	XA
		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
10	D-T	322 ± 23‡	0	0	0	0	188 ± 37	255 ± 36	0	
17	DL-T	240 ± 31	109 ± 20	70 ± 16	58 ± 29	89 ± 12	378 ± 22	140 ± 24	0	28 ± 11
11	L-T	202 ± 63	201 ± 17	108 ± 27	108 ± 21	124 ± 20	694 ± 45	49 ± 16	0	58 ± 13
8	D-K	470 ± 46		0		0	329 ± 21	0	108 ± 17	
8	L-K	177 ± 39		74 ± 31	96 ± 22	185 ± 46	202 ± 45	0	354 ± 21	43 ± 19

* For abbreviations used and amounts of substrate and conditions of tests, see Table I.

† Liver slices failed to produce IPA in measurable amounts from T. Kidney slices failed to produce K from T or to yield AA from any substrate.

‡ ± = Standard deviation of the mean.

either kynurenic acid or anthranilic acid from D-kynurenine, but the normal liver slice formed a small amount of kynurenic acid from D-tryptophan.

The results obtained with L-tryptophan correlate reasonably well with the reported increases in urinary output of kynurenine, xanthurenic acid, and kynurenic acid (1, 3) and in the urinary excretion of anthranilic acid (2, 3) in the riboflavin-deficient rat after the administration of L-tryptophan. The greater production of xanthurenic acid from L-kynurenine in tissue slices from the riboflavin-deficient rat fails to agree with the small excretion reported to occur in the deficient animal after the injection of L-kynurenine (4). Failure of the normal liver or kidney slice to produce xanthurenic acid from L-tryptophan and L-kynurenine is in accord with previous observations (15). If xanthurenic acid derivatives were produced in our incubation mixtures from L-kynurenine (15), they did not respond to the colorimetric assay for xanthurenic acid. Incidentally, Price and Dodge (24) have noted that the original neutral solvent system of Mason and Berg (15) gives the same R_F for xanthurenic acid as for kynurenic acid. They identified the more brilliant chromatographic spot which Mason and Berg had mistakenly associated with free xanthurenic acid as the somewhat more rapidly migrating 8-methyl ether of xanthurenic acid (24).

The normal liver slice yielded considerably more indolepyruvic acid (about 80 per cent more) from L-tryptophan than from D-tryptophan. None was found in analogous tests with liver slices from the riboflavin-deficient rat. The accumulation of indolepyruvic acid in media which had been incubated with normal kidney slices was markedly greater with D-tryptophan than with the L-isomer. Although production persisted in kidney slices from rats that were riboflavin-deficient, the accumulation was only a third or fourth as great.

The formation of indolepyruvic acid from L-tryptophan could presumably have been effected either by transaminase or by the riboflavin phosphate-containing L-amino acid oxidase. More active preparations of L-amino acid oxidase have been obtained from rat kidney than from rat liver (25). Although more flavin adenine dinucleotide is present per unit weight in rat liver than in rat kidney (26), the D-amino acid oxidase activity of the former is only an eighth as great, or even less (27). No other enzyme has been found to convert D-amino acids to the corresponding α-keto acids in animal tissue.

TABLE III

Influence of riboflavin upon conversion of D-, DL-, and L-tryptophan to indolepyruvic acid by liver and kidney slices from riboflavin-deficient rats

No. of rats	Tryptophan (1000 μg.)	Indole pyruvic acid production			
		Liver slices*		Kidney slices*	
		Alone	Plus 0.5 mg. of riboflavin	Alone	Plus 0.5 mg. of riboflavin
		μg.	μg.	μg.	μg.
5	D	0	63 ± 12†	294 ± 22	798 ± 37
5	DL	0	49 ± 7	133 ± 19	448 ± 58
5	L	0	91 ± 10	48 ± 8	198 ± 14

* 1000 μg. of substrate in 3 ml. of Krebs-Henseleit buffer at pH 7.4, incubated with 400 mg. of tissue slices for 3 hours at 37° in an O_2 atmosphere.

† ± = Standard deviation of the mean.

Table III shows that the capacity of liver and kidney slices from the riboflavin-deficient rat to produce indolepyruvic acid could be restored, apparently fully, by the addition of riboflavin to the media.

Table IV presents evidence that indolepyruvic acid undergoes appreciable conversion to tryptophan upon incubation with either the liver or the kidney slice, and that incubation with the former also yields kynurenine. The riboflavin deficiency did not enhance significantly either the tryptophan or the kynurenine accumulation, but considerably less indolepyruvic acid was recovered from the media incubated with tissue from the riboflavin-deficient animal. The data substantiate the assumption that indolepyruvic acid can be utilized for growth in the rat (28, 9) fed no tryptophan because it is converted into L-tryptophan; hence also the assumption that D-tryptophan becomes available for growth (29, 30) by inversion through indolepyruvic acid. Evidence that rat kidney does convert D-tryptophan to L-tryptophan has been presented by Kotake and Goto (31).

Inversion of D-tryptophan by the normal liver slice could conceivably account also for its conversion to kynurenic acid (*cf.* Table I). Failure to detect kynurenine and anthranilic acid may reflect a smaller sensitivity of the tests for these pro-

TABLE IV

*Production of tryptophan and kynurenine upon incubation of indolepyruvic acid with liver and kidney slices from normal and riboflavin-deficient rats**

Type and No. of Rats	Liver slice tests†			Kidney slice tests		
	IPA recovered	T found	K found	IPA recovered	T found	K found
	μg.	μg.	μg.	μg.	μg.	μg.
Normal (5)	583 ± 40‡	252 ± 37	58 ± 28	652 ± 26	179 ± 13	0
Riboflavin-deficient (9)	135 ± 13	263 ± 37	69 ± 16	267 ± 21	190 ± 19	0

* For abbreviations used see Table I. 1000 μg. of IPA incubated with 400 mg. of tissue slices in 3 ml. of Krebs-Henseleit buffer, pH 7.4 at 37° for 3 hours in an O_2 atmosphere.

† Weak spots of XA, KA, and AA were detected on some chromatograms. XA and KA were not estimated because insufficient medium remained for suitable analysis. Eluates of the AA spots yielded traces too small for accurate estimation.

‡ ± = Standard deviation of the mean.

ducts, but, more likely, differences in relative rates of the various reactions involved in the conversion of D-tryptophan to indolepyruvic acid, to L-tryptophan, to L-kynurenine, and thence to kynurenic acid or anthranilic acid. Weak spots for kynurenine were detected on the chromatograms in a few instances, in two of which 4 μg. were measured. In four tests, definite anthranilic acid spots were revealed by ultraviolet fluorescence, but the quantities present were too small for estimation.

The greater accumulation of indolepyruvic acid in the tryptophan tests with normal liver was inadequate to account fully for the smaller accumulation of the other metabolites noted in these media. The possibility of metabolism by other routes is, of course, not precluded. The considerably larger accumulation of indolepyruvic acid also in L-tryptophan media incubated with kidney slices from the normal as compared with the riboflavin-deficient rat leads one to suggest that its production in the normal intact rat may simply impair the efficiency of conversion of L-tryptophan to kynurenine, kynurenic acid, and anthranilic acid; may promote metabolism by routes available only to indolepyruvic acid; or may lead to the elimination of indolepyruvic acid in the urine.

In no instance did the metabolites produced and the substrates recovered in the experiments recorded in any of the tables fully account for the substrates employed. Some destruction of substrate could be shown to occur during incubation in the absence of the tissue slices, but this did not exceed 10 per cent, hence was far too small to account for the incomplete recovery.

Mason observed that, in a nitrogen atmosphere, homogenates of normal kidney catalyzed the conversion of L-kynurenine to kynurenic acid, presumbly by transamination, but yielded very much less kynurenic acid from D-kynurenine under analogous conditions (32). Conversion of D-kynurenine to *o*-aminobenzoylpyruvic acid by D-amino acid oxidase might well have accounted for the greater production of kynurenic acid by the kidney slice in our tests in which an oxygen atmosphere was employed. The yield of kynurenic acid from D-kynurenine was not measurable with liver tissue (Table I) in which D-amino acid oxidase is much less active, nor was it increased appreciably with kidney from the riboflavin-deficient rat (Table II). Analogous tests with L-kynurenine showed an appreciable accumulation of kynurenic acid, even with liver tissue, and an approximately doubled yield with liver and kidney from the riboflavin-deficient animal.

The optimal pH for D-amino acid oxidase has been reported to be 8.2 to 8.4 (22), but at pH 8.4 flavin adenine dinucleotide undergoes more rapid degradation than at pH 7.3 (33). In a series of tests made with normal kidney slices at pH 8.2, in which sodium pyrophosphate was used as the buffer (12) but all other conditions were the same as in the studies recorded in Table I, the indolepyruvic acid found after incubation with tissue from 6 rats was only 460 ± 35 μg. with the D-tryptophan, 196 ± 28 μg. with DL-tryptophan, and 52 ± 15 μg. with L-tryptophan. Corresponding figures with kidney from 9 riboflavin-deficient rats were also lower: 102 ± 31, 68 ± 22, and 0 μg., respectively.

SUMMARY

The influence of riboflavin deficiency upon the metabolism of tryptophan was studied by comparing metabolite accumulation in media incubated at pH 7.4 with liver and kidney slices from the deficient rat with the accumulation when tissues from the normal animal were employed.

Indolepyruvic acid accumulation was considerably less in the tests with liver and kidney slices from the riboflavin-deficient rat. Addition of riboflavin to the media increased the indolepyruvic acid production, but the deficiency exerted little, if any, influence upon the capacities of the liver and kidney slices to convert indolepyruvic acid to tryptophan, as judged by the accumulation of the latter in the media.

The accumulation of kynurenine, kynurenic acid, and anthranilic acid was greater in the L-tryptophan media incubated with liver slices from the riboflavin-deficient animal. The deficiency stimulated the production of xanthurenic acid by both the liver and the kidney slice from L-tryptophan and L-kynurenine. It increased the production of kynurenic acid and anthranilic acid from L-kynurenine.

No kynurenic acid or anthranilic acid was produced by either the normal or deficient liver from D-kynurenine. A small amount of kynurenic acid was formed in the normal liver from D-tryptophan, possibly after its initial inversion. No kynurenic acid was produced from tryptophan by either the normal or the deficient kidney slice. It was produced by both from D- and L-kynurenine. Riboflavin deficiency greatly increased its production by the kidney slice from L-kynurenine, but only slightly, if even significantly, its production from D-kynurenine.

Correlation of the results obtained in these tests with the urinary output of tryptophan metabolites in the intact riboflavin-deficient animal is discussed.

REFERENCES

1. Porter, C. C., Clark, I., and Silber, R. H., *Arch. Biochem.*, **18**, 339 (1948).
2. Mason, M., *J. Biol. Chem.*, **201**, 513 (1953).
3. Charconnet-Harding, F., Dalgliesh, C. E., and Neuberger, A., *Biochem. J.*, **53**, 513 (1953).
4. Henderson, L. M., Koski, R. E., and D'Angeli, F., *J. Biol. Chem.*, **215**, 369 (1955).
5. Shabica, A. C., and Tishler, M. J., *J. Am. Chem. Soc.*, **71**, 3251 (1949).
6. Warnell, J. L., and Berg, C. P., *J. Am. Chem. Soc.*, **76**, 1708 (1954).
7. Kallio, R. E., and Berg, C. P., *J. Biol. Chem.*, **181**, 333 (1949).
8. Boyd, W. J., and Robson, W., *Biochem. J.*, **29**, 555 (1935).
9. Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 219 (1929).
10. Jones, J. H., and Foster, C., *J. Nutrition*, **24**, 245 (1942).
11. Krebs, H. A., and Henseleit, H., *Z. physiol. Chem.*, **210**, 33 (1932).
12. Bender, A. E., and Krebs, H. A., *Biochem. J.*, **46**, 210 (1950).
13. Stowe, B. B., and Thimann, K. V., *Arch. Biochem. Biophys.*, **51**, 499 (1954).
14. Bentley, J. A., Farrar, K. R., Housley, N., Smith, G. F., and Taylor, W. C., *Biochem. J.*, **64**, 44 (1956).
15. Mason, M., and Berg, C. P., *J. Biol. Chem.*, **188**, 783 (1951).
16. Warnell, J. L., Ph.D. Dissertation, State University of Iowa, 1955.
17. Sealock, R. R., *Science*, **94**, 73 (1941).
18. Cavallini, D., and Frontali, N., *Biochim. et Biophys. Acta*, **13**, 440 (1954).
19. Mason, M., and Berg, C. P., *J. Biol. Chem.*, **195**, 515 (1952).
20. Brown, F. C., and Berg, C. P., *J. Biol. Chem.*, **221**, 1083 (1956).
21. Tabone, J., and Magis, C., *Bull. soc. chim. biol.*, **28**, 744 (1946).
22. Tabone, J., Roberts, D., Thomassey, S., and Mamounas, N., *Bull. soc. chim. biol.*, **32**, 529 (1950).
23. Glazer, H. S., Mueller, J. F., Thompson, C., Hawkins, V. R., and Vilter, R. W., *Arch. Biochem. Biophys.*, **33**, 243 (1951).
24. Price, J. M., and Dodge, L. W., *J. Biol. Chem.*, **223**, 699 (1956).
25. Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **155**, 421 (1944); **161**, 583 (1945).
26. Negelein, E., and Bromel, H., *Biochem. Z.*, **300**, 225 (1935).
27. Birkofer, L., and Wetzel, R., *Z. physiol. Chem.*, **264**, 31 (1940).
28. Jackson, R. W., *J. Biol. Chem.*, **84**, 1 (1929).
29. du Vigneaud, V., Sealock, R. R., and Van Etten, C., *J. Biol. Chem.*, **98**, 565 (1932).
30. Berg, C. P., *J. Biol. Chem.*, **104**, 373 (1934).
31. Kotake, Y., and Goto, S., *Z. physiol. Chem.*, **248**, 41 (1937); **270**, 48 (1941).
32. Mason, M., *J. Biol. Chem.*, **211**, 839 (1954).
33. Krebs, H. A., *Biochem. J.*, **29**, 1620 (1935).

The Source and State of the Hydroxylysine of Collagen

II. FAILURE OF FREE HYDROXYLYSINE TO SERVE AS A SOURCE OF THE HYDROXYLYSINE OR LYSINE OF COLLAGEN*

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The amino acid 5-hydroxylysine, first isolated from gelatin (2),[1] has been found absent in all types of proteins other than collagen that have been examined (3). It is not an essential amino acid for animal nutrition (4), and hence must be formed from other material in the animal body.

Previous papers from this laboratory (5, 6) have shown that lysine is the chief source of the hydroxylysine incorporated into collagen. When uniformly C^{14}-labeled lysine was administered to young rats, either with food (5) or by injection (6), both lysine and hydroxylysine in the skin collagen were labeled to an approximately equal extent, indicating that lysine serves as the chief, and probably only significant, source of hydroxylysine in rat skin collagen. The relative rates of labeling of the two amino acids indicated that the hydroxylation of lysine to form hydroxylysine occurs simultaneously, or nearly so, with incorporation into the collagen (6). The results were confirmed by Piez and Likins (7), with regard to rat collagen of skin, tail, bone, and dentin, and by Kao and Boucek (8) with regard to collagen formed in implanted polyvinyl sponges.

The questions remained, whether administered free hydroxylysine can either be incorporated into the hydroxylysine or collagen, or be dehydroxylated, by reversion of the process by which lysine is hydroxylated, and incorporated into the lysine of collagen. With regard to the latter question, the probability of a negative answer is indicated by a feeding experiment by Lindstedt (9). He found that synthetic DL-hydroxylysine (¼ allo isomer) could not replace an equimolar amount of L-lysine in maintaining the growth of rats. The possibility remained, however, that the hydroxylation of lysine might be to some extent reversible.

The object of the work reported in the present paper has been to ascertain by isotope analysis whether hydroxylysine administered either orally or by injection to growing rats is incorporated into either the hydroxylysine or the lysine of collagen. Tritium-labeled hydroxylysine has been administered to young rats and the extent of incorporation of the label into the lysine and hydroxylysine of the skin collagen has been measured. The amount of tritium found in either the hydroxylysine or lysine of the collagen was so slight that it does not appear that free hydroxylysine is to a significant extent incorporated into collagen as either hydroxylysine or lysine.

The results are consistent with the deduction of Piez and Likins (7), who observed that the C^{14} content of the hydroxylysine isolated from collagen after injection of C^{14}-labeled lysine into rats was not altered by accompanying injections of unlabeled hydroxylysine. Piez and Likins reasoned that "if preformed, unbound hydroxylysine can be incorporated into collagen, the presence of large amounts of the free amino acid would decrease the contribution of lysine-C^{14} to collagen hydroxylysine."

EXPERIMENTAL

Two experiments were carried out. In each experiment tritium-labeled hydroxylysine was given to four young rats, and the relative extents of incorporation of labeled material into the lysine and hydroxylysine of the skin collagen were measured.

In Experiment I (Table I) the labeled hydroxylysine was administered to rats of 50 to 60 gm. weight by addition to the daily diet for 2 weeks before the rats were killed. The hydroxylysine was a synthetic preparation labeled with tritium attached to carbon 6.

In Experiment II (Table II) the labeled hydroxylysine was given by a single intraperitoneal injection and the rats were killed 4 hours later. The hydroxylysine was a preparation that had been tritiated by the method of Wilzbach (10), and was more radioactive than that used in Experiment I.

Experiment I

Preparation of Hydroxylysine Labeled with Tritium Attached to C-6—DL-Hydroxylysine was prepared by the method of Touster (11) as modified by Lindstedt (12). However, instead of using $KC^{14}N$, as was done by Lindstedt to label the carbon, we employed tritium gas for reduction of the terminal —CH(OH)·CN group to —CH(OH)—CH_2 (NH_2). Tritium is introduced by the reduction into the —CH_2·NH_2 group and, by exchange, into the OH group, but in the NH_2 and OH groups is replaced by H^1 in subsequent operations in water solution, leaving H^3 stably bound only in the CH_2 group. The hydroxylysine obtained

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[1] We take occasion to correct a figure in the paper (2) in which the isolation of hydroxylysine in crystalline form, as picrate and monochloride, was first reported. It was stated that hydroxylysine when analyzed for amino nitrogen by the nitrous acid method (3) reacted with 95 per cent of its nitrogen in 3 minutes at 25°, under conditions that give 100 per cent reaction with the α amino groups of the amino acids. Van Slyke and Plazin (unpublished results) find that hydroxylysine entirely free from lysine reacts with 100 per cent of its nitrogen in 3 minutes at 25°, the ϵ amino nitrogen reacting as rapidly as the α-nitrogen.

was presumably a mixture of equal amounts of DL-*n*- and DL-allohydroxylysine. The specific activity of the preparation was 43.8 μc. per mmole.

Treatment of Rats—A rat, weighing 61 gm. at the start, consumed in 2 weeks 250 gm. of the "basal" zein diet of Henderson *et al.* (13), referred to by them as supplement B, to which 2.5 gm. of unlabeled lysine and 0.134 gm. of tritium-labeled synthetic DL-hydroxylysine hydrochloride were added. On this diet, the rat grew 43 gm. in the 2 weeks. The approximate amount of hydroxylysine incorporated is estimated by assuming that 35 per cent of the growth was dry solids, of the solids ⅛ was protein, of the protein ¼ was collagen, and of the collagen 0.01 was L-hydroxylysine. The incorporated L-hydroxylysine thus estimated is 17 mg. The amount of L-*n*-hydroxylysine in the food consumed was 33.8 mg.

The conditions of the experiment were similar to those of our previous experiments (5), in which labeled lysine was fed, except that in the present experiment it is the hydroxylysine that is labeled.

Preparation and Analysis of Skin Collagen—The preparation of gelatin from skin collagen previously described (6) was followed, with precipitation of nongelatin proteins from the gelatin solution by trichloroacetic acid. The gelatin was not precipitated with acetone. Hydrolysis of gelatin and precipitation of the basic amino acids were carried out as previously described (5). The basic amino acids were chromatographed on Dowex 50 in 0.1 M citrate at pH 5. This procedure, as shown by Hamilton (14) gives good separation of lysine and hydroxylysine from each other and from histidine and arginine. The solutions of lysine and hydroxylysine were desalted with Dowex 50 as previously described (6) and eluted in 6 N HCl, which was removed by concentration in a vacuum and drying over KOH. The dihydrochlorides of lysine and hydroxylysine thus obtained were freed of chloride by treatment with silver sulfate, followed by barium hydroxide to remove SO_4, and by CO_2 to remove barium. To the solution of each amino acid thus obtained exactly enough picric acid was added to form the monopicrate, which was crystallized by concentrating the solution. Samples of the picrates were analyzed for tritium by the method of Christman (15), in which the hydrogen is set free by heating with metallic zinc and is counted in the gas counter of Bernstein and Ballantine (16).

Experiment II

Preparation of Tritium-Labeled Hydroxylysine—In order to obtain hydroxylysine of greater specific activity for the injection experiment synthetic DL-hydroxylysine was treated with tritium gas according to the procedure of Wilzbach (10). A gm. of DL-hydroxylysine was exposed to 1.2 curies of tritium gas at room temperature and a pressure of 0.33 atmosphere for 14 days.[2] In order to remove labile tritium the resulting material was dissolved in 5 successive portions of 6 ml. each of distilled water, and each portion of water was removed by evaporation under a vacuum at room temperature.

Chromatography in phosphate buffer by the procedure of Hamilton and Anderson (17) gave a preparation that contained approximately 70 per cent of DL-*n*-hydroxylysine and 30 per cent of the allo isomers.

This material, 10 mg., was rechromatographed and the phosphate buffer removed according to the procedure of Mueller *et al.* (18). Isolation and counting of the sample of monopicrate indicated that the specific activity was 9.62×10^3 μc. per mmole of hydroxylysine.

[2] The preparation was carried out by Dr. Seymour Rothstein of the New England Nuclear Corporation, Boston, Massachusetts.

TABLE I

Proportions of lysine and hydroxylysine in skin collagen derived from labeled lysine or hydroxylysine in food

Labeled amino acids in food				Labeled amino acids in skin collagen			
Amino acid	Specific activity of pure amino acid (*a*)	L-Normal amino acid in 100 gm. of food*	Duration of feeding	Specific activity		Derived from labeled amino acid in food	
				Hydroxylysine (*b*)	Lysine (*c*)	Hydroxylysine 100 *b*/*a*	Lysine 100 *c*/*a*
	μc./mmole	mmoles	days	μc./mmole	μc./mmole	%	%
DL-Hydroxylysine	95.8	0.068	14	4.10×10^{-3}	3.78×10^{-3}	0.0042	0.0041
L-Lysine†	4.83	6.84	21	0.98	0.98	20.3	20.3

* Amount of L-*n*-hydroxylysine is calculated as ¼ the DL-isomer mixture.

† The lysine data are from Sinex and Van Slyke (5).

TABLE II

Proportions of lysine and hydroxylysine in skin collagen derived from labeled amino acids intraperitoneally injected

Labeled amino acid injected				Labeled amino acids in skin collagen			
Amino acid	Specific activity (*a*)	Amount injected per rat		Specific activity		Derived from injected amino acid	
		Total	L-Normal	Hydroxylysine (*b*)	Lysine (*c*)	Hydroxylysine 100 *c*/*a*	Lysine 100 *c*/*a*
	μc./mmole	mmole	mmole	μc./mmole	μc./mmole	%	%
DL-Hydroxylysine*	9.62×10^3	0.0103	0.0036	2.55×10^{-3}	2.40×10^{-3}	2.63×10^{-4}	2.50×10^{-4}
L-Lysine†	153	0.105	0.105	0.518	0.732	0.34	0.48

* Preparation with 70 per cent DL-*n*-isomer, 30 per cent DL-allo isomer.

† From data on 4-hour rats of Van Slyke and Sinex (6).

Treatment of Rats—Hydroxylysine, 6.68 mg., in four approximately equal portions was injected intraperitoneally into four rats of 60 to 66 gm. weight. Since 70 per cent of the hydroxylysine in the preparation was the DL-*n* form, it is calculated that each rat received 0.59 mg. of L-*n*-hydroxylysine. The rats were killed 4 hours after the injection.

Preparation and Analysis of Skin Collagen—The procedure was the same as in Experiment I, except that in Experiment II unlabeled DL-hydroxylysine was added to the gelatin solution to produce a concentration of 0.15 per cent of hydroxylysine in the solution before treatment of the latter with trichloroacetic acid. The addition was made with the thought that the free unlabeled hydroxylysine might displace from the gelatin any of the highly active tritium-labeled hydroxylysine that might have been adsorbed or otherwise nonspecifically bound by the collagen *in vivo* from the injected labeled hydroxylysine (however, the work of Cornwall and Luck (19) appearing after these experiments

were done indicates that addition of the amino acid before dialysis probably had no influence on the results). In order to insure complete removal of the added inactive hydroxylysine, the gelatin solution was dialyzed against 5 successive changes of distilled water instead of the 3 changes used previously (6) and in Experiment I.

Preparation of the picrates of lysine and hydroxylysine from the hydrolyzed collagen, and determination of their activities by Christman's method were as in Experiment I.

DISCUSSION

Results of Experiment I. Administration of Labeled Hydroxylysine in Diet

The results of feeding labeled hydroxylysine are shown in the upper row of figures in Table I. For comparison the results of feeding labeled lysine previously reported (5) are shown in the lower row of figures in the table. The percentages of the lysine and hydroxylysine residues of skin collagen derived from the labeled amino acids in the food are calculated as

$$\frac{\text{specific activity of amino acid isolated}}{\text{specific activity of amino acid fed}} \times 100.$$

The results show that incorporation of fed hydroxylysine into skin collagen was insignificant compared with incorporation of fed lysine. After feeding lysine the activities of both the lysine and the hydroxylysine of the collagen were 20.3 per cent of the activity of the fed lysine, whereas after feeding hydroxylysine the activities of both the lysine and hydroxylysine in the collagen were only 0.004 per cent of the activity of the fed hydroxylysine.

The fact that after feeding labeled hydroxylysine the specific activities of lysine and hydroxylysine in the collagen were equal could be interpreted to indicate that about $\frac{8}{9}$ of the incorporated hydroxylysine was reduced to lysine during the process of incorporation, a reversal of the hydroxylation of lysine that was noted after either feeding (5) or injecting (6) labeled lysine. However, the results of injecting hydroxylysine in Experiment II do not support such an interpretation. It appears more probable that a small part of the fed hydroxylysine was reduced to lysine by bacterial action in the intestine, and that this lysine was the source of the small amounts of labeled lysine and hydroxylysine that were incorporated into the collagen.

Correction for the effect of the greater amount of labeled lysine fed does not alter the significance of the difference in incorporation between the two amino acids. The amount of L-hydroxylysine in the hydroxylysine feeding experiment was only $\frac{1}{100}$ of the amount of L-lysine in the lysine feeding experiment, but was equivalent, as a source of collagen hydroxylysine, to $\frac{1}{11}$ the amount of lysine fed, because only $\frac{1}{9}$ of incorporated lysine is changed to hydroxylysine (5, 6). If hydroxylysine were incorporated to the same extent as lysine per unit of each present, the percentage of labeled collagen hydroxylysine derived from fed hydroxylysine would therefore be $\frac{1}{11}$ the percentage derived from fed lysine. However, the percentage, 0.004, in the hydroxylysine feeding (upper row, Table I) is only $\frac{1}{5000}$ the percentage, 20.5, in the lysine feeding (lower row, Table I).

Results of Experiment II. Intraperitoneal Injection of Labeled Hydroxylysine

In Table II the results of labeled hydroxylysine injection in Experiment II are given in the upper row of figures. In the lower row are given for comparison the results of a previous experiment (6) in which labeled lysine was injected. In both experiments the rats were killed 4 hours after the injections.

The incorporation of labeled injected hydroxylysine into the collagen was quantitatively insignificant compared with the incorporation of labeled lysine. Correcting for the difference in amounts of labeled L-lysine and L-hydroxylysine injected, as in the discussion of Experiment I, one would expect the percentage of collagen hydroxylysine derived from injected hydroxylysine to be $\frac{1}{12}$ as great as the percentage of collagen lysine derived from injected lysine, if the same proportion of injected hydroxylysine as of lysine were incorporated. Actually after injection of the labeled hydroxylysine the percentage of collagen hydroxylysine derived from the injected material was only $\frac{1}{1300}$ as great as after injection of labeled lysine.

The results (upper row, Table II) indicate that a slight but measurable amount of the injected hydroxylysine was taken up directly by the skin collagen. This conclusion is indicated by the fact that the specific activity of the hydroxylysine isolated from the collagen was about 10 times the specific activity of the isolated lysine. This activity ratio excludes the possibility that the labeled hydroxylysine found in the collagen was formed, as suggested for fed hydroxylysine in Experiment I, via intestinal reduction to lysine and incorporation of the resultant lysine accompanied by its partial hydroxylation. It is possible that the small amount of labeled hydroxylysine found in the collagen was taken up by a nonspecific adsorption or combination of the amino acid with the collagen such as that studied *in vitro* by Cornwall and Luck (19). These authors have shown that the proteins, histone and insulin, in solution can form combinations with small amounts of phenylalanine and lysine that are not dissociated by precipitation and dialysis. Whatever the mechanism of the observed combination of collagen with injected hydroxylysine, the proportion combined is too slight to indicate that direct incorporation of free hydroxylysine can serve as a physiologically significant source of collagen hydroxylysine.

SUMMARY

Feeding or injecting radioactive hydroxylysine into young rats did not lead to the incorporation of significant amounts into the hydroxylysine of the skin collagen. Slight amounts of radioactive hydroxylysine and lysine were found in the collagen hydrolysates, but the amounts were so small, compared with those obtained after similar administration of radioactive lysine, that they excluded free hydroxylysine as a physiologically significant source of either hydroxylysine or lysine in the collagen.

The results reinforce the conclusion reached by the authors from experiments with labeled lysine (5, 6), that the hydroxylysine of collagen in the rat originates from hydroxylation of lysine during formation of the collagen.

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REFERENCES

1. Sinex, F. M., and Van Slyke, D. D., *Federation Proc.*, **16**, 250 (1957).
2. Van Slyke, D. D., Hiller, A., Dillon, R. T., and MacFadyen, D. A., *Proc. Soc. Exptl. Biol. Med.*, **38**, 548 (1938).
3. Hamilton, P. B., and Anderson, R. A., *J. Am. Chem. Soc.*, **77**, 2892 (1955).
4. Rose, W. C., *Science*, **86**, 2231 (1937); *Nutrition Revs.*, **8**, 118 (1950).
5. Sinex, F. M., and Van Slyke, D. D., *J. Biol. Chem.*, **216**, 245 (1955).
6. Van Slyke, D. D., and Sinex, F. M., *J. Biol. Chem.*, **232**, 797 (1958).
7. Piez, K. A., and Likins, R. C., *J. Biol. Chem.*, **229**, 101 (1957).
8. Kao, K. Y. T., and Boucek, R. J., *Proc. Soc. Exptl. Biol. Med.*, **98**, 526 (1958).
9. Lindstedt, S., *Acta Physiol. Scand.*, **27**, 377 (1952).
10. Wilzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013 (1957).
11. Touster, O., *J. Am. Chem. Soc.*, **73**, 491 (1951).
12. Lindstedt, S., *Acta Chem. Scand.*, **7**, 340 (1953).
13. Henderson, L. M., Koeppe, O. J., and Zimmerman, H. H., *J. Biol. Chem.*, **201**, 697 (1953).
14. Hamilton, P. B., in C. Calmon and T. R. E. Kressman (Editors), *Ion exchangers in organic and biochemistry*, Interscience Publishers, Inc., New York, 1957, p. 259.
15. Christman, D. R., *Chemist Analyst*, **41**, 5 (1957).
16. Bernstein, W., and Ballantine, R., *Rev. Sci. Instr.*, **21**, 158 (1950).
17. Hamilton, P. B., and Anderson, R. A., *J. Biol. Chem.*, **213**, 249 (1955).
18. Mueller, G. C., Bowman, G., and Herranen, A., *Anal. Chem.*, **27**, 1357 (1955).
19. Cornwall, D. G., and Luck, J. M., *Arch. Biochem. Biophys.*, **73**, 391 (1958).

The Distribution of Glutamic-γ-Aminobutyric Transaminase in the Nervous System of the Rhesus Monkey

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Respiration of brain tissue is supported equally well by glucose, glutamic acid, γ-aminobutyric acid, and succinic semialdehyde *in vitro* (1, 2). The last three metabolites may be related in a common metabolic pathway involving glutamic-γ-aminobutyric transaminase (3), glutamic decarboxylase (4), and semialdehyde dehydrogenase (5). There is little evidence for the occurrence of this pathway in mammalian tissues other than in the central nervous system (6). The possibility that the occurrence of these enzymes may relate specifically to neuronal function has prompted the present quantitative histochemical survey of the distribution of glutamic-γ-aminobutyric transaminase.

EXPERIMENTAL

Materials and Methods

Succinic semialdehyde was prepared from diethylformyl succinate (7). A sample of diethylformylsuccinate was the gift of Dr. E. C. Layne.

Frozen-dried sections of various areas of the nervous system of the rhesus monkey were prepared according to the procedure of Lowry (8). Samples were dissected under a stereomicroscope with histological control from adjacent sections stained with cresyl violet. Sample weights were obtained with a quartz fiber balance which had a sensitivity of 1.45 μg. per mm. of deflection.

The transaminase assay was based upon the measurement of succinic semialdehyde produced from γ-aminobutyrate during incubation of the enzyme. The succinic semialdehyde was measured fluorometrically by a procedure based on a reaction described by Velluz *et al.* (9). The specificity of this reaction permits the measurement of α-methylenic aldehydes in the presence of keto-acids. The reagent, 3,5-diaminobenzoic acid, condenses with α-methylenic aldehydes to form quinaldine derivatives. A specific fluorometric method for deoxyribose nucleic acids which is based upon the same principle has been recently described (10).

Procedure for Measurement of Succinic Semialdehyde—The reagent was a 0.25 M solution of 3,5-diaminobenzoic acid (Aldrich Chemical Company) adjusted to pH 6 with K_2CO_3. Equal volumes of sample and reagent were mixed and heated for 1 hour in a water bath at 60°. A 1- to 5-μl. aliquot of the reaction mixture was diluted with 1.0 ml. of glass-distilled water (pH 5) and the fluorescence of the diluted aliquot was measured with a photomultiplier fluorometer (Farrand model A). Primary excitation was obtained from the 405 mμ Hg line (405 mμ interference filter + Corning No. 5113 glass filter) and the secondary emission was measured at 505 mμ (505 mμ interference filter + Corning No. 3387 glass filter). The fluorescence characteristics of the reagent and the product are shown in Fig. 1.

With the suggested filters, the reagent blank was equivalent to a concentration of about 10^{-4} M succinic semialdehyde in the original sample. Thus 10^{-10} moles of succinic semialdehyde may be measured. The reagent blank relative to sample fluorescence may be reduced about 3-fold by decreasing the reagent concentration to 0.1 M. However, less than maximal fluorescence is developed under these conditions. It did not seem advisable to recrystallize the reagent, since the small amount of dinitrobenzoate which contaminates the reagent was found to accelerate the rate of development of fluorescence.

Standard solutions of succinic semialdehyde were prepared by refluxing 1 ml. of the diethyl ester of formylsuccinate with 10 ml. of 0.2 N HCl for 2 hours. The stock solution so obtained was stable for several months when kept frozen. The concentration of succinic semialdehyde in these solutions was found to be 0.05 to 0.07 M by measuring the reduction of pyridine nucleotides with semialdehyde dehydrogenases obtained from monkey brain (5) or from *Pseudomonas fluorescens* (11).

Procedure for Measurement of Glutamic-γ-aminobutyric Transaminase in μg. Samples of Monkey Brain—A buffered substrate solution was prepared containing 0.1 M α-ketoglutaric acid and 0.25 M γ-aminobutyric acid adjusted to pH 8.4 with NaOH. Samples of lyophilized tissue (3 to 15 μg. dry weight) were placed in 3-mm. diameter test tubes in an ice bath and 5 μl. of the buffered substrate was added to each with a micropipet (12). Incubation was carried out in a water bath at 38° for 2 hours. A standard curve of succinic semialdehyde concentration *versus* fluorescence was obtained for each set of samples. The stock solution of succinic semialdehyde was diluted with the buffered substrate solution to concentrations of 5 to 20 × 10^{-4} M just before use. Aliquots, 5 μl., of these standards were carried through the procedure in the same manner as the samples.

RESULTS

Experiments with monkey brain homogenates were carried out to establish the optimal conditions for the enzyme assay (13)

* This work was performed under the tenure of a postdoctoral fellowship of the National Institute of Neurological Diseases and Blindness.

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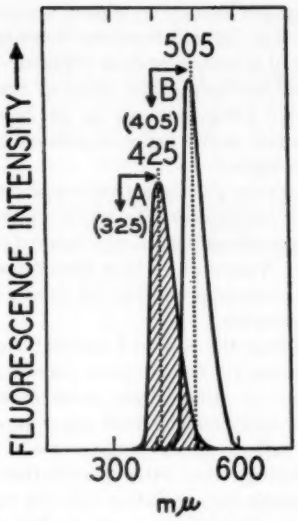

FIG. 1. Fluorescence spectra and excitation maxima of 3,5-diaminobenzoic acid (*A*) and of the condensation product with succinic semialdehyde (*B*). The data represented in the figure were obtained with an Aminco-Bowman spectrophotofluorimeter.

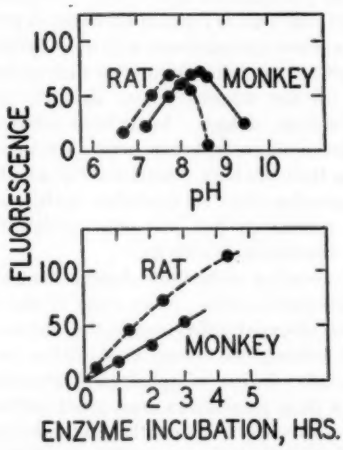

FIG. 2. Transaminase activity as a function of pH and of incubation time at 38° in homogenates of rat and monkey brain tissues. Individual points on the curves are the average of triplicate samples. The determinations were carried out as indicated in the text.

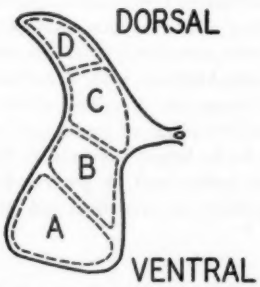

FIG. 3. Dissection of samples from the gray matter of spinal cord. Data in Table III were obtained from samples from the indicated areas.

TABLE I

Distribution of glutamic-γ-aminobutyric transaminase activity in cortical structures

	mmoles/kg./hour
Occipital cortex	
Layer 1	30 ± 11
Layer 2 + 3	55 ± 9
Layer 4	40 ± 8
Layer 5 + 6	9 ± 1
Subjacent white	2 + 1
Motor cortex	
Layer 1	34 ± 6
Layer 2 + 3	65 ± 11
Layer 5 + 6	36 ± 2
Subjacent white	4 + 1
Hippocampus	
Molecularis	94 ± 9
Radiata	66 ± 6
Pyramidalis	68 ± 10
Alveus + oriens	46 ± 2
Cerebellar cortex	
Molecular layer	150 ± 9
Granular layer	74 ± 10

TABLE II

Distribution of glutamic-γ-aminobutyric transaminase activity in subcortical structures

	mmoles/kg./hour
Optic tract	0
Pituitary stalk	0
Fasciculus gracilis	0
Lateral pyramids	0
Globus pallidus	52 ± 3
Lateral thalamic nucleus	53 ± 5
Red nucleus	62 ± 11
Midbrain central gray	94 ± 14
Substantia nigra	96 ± 9
Putamen	105 ± 4
Reticular formation	113 ± 21
Caudate nucleus	114 ± 14
Medial thalamic nucleus	115 ± 7
Posterior hypothalamus	124 ± 19
Superior colliculus, inner gray	137 ± 20
Anterior hypothalamic nucleus	149 ± 16
Abducens nucleus	209 ± 18
Inferior colliculus	219 ± 14
Dentate nucleus	252 ± 11
Superior colliculus, ext. and middle gray	276 ± 7
Inferior olivary nucleus	294 ± 7

(Fig. 2). Under these conditions, the transamination rate in whole monkey brain homogenate was found to be approximately 150 mmoles per kg. per hour[1] in terms of protein content.

The quantitative data describing the distribution of glutamic-γ-aminobutyric are summarized in Tables I to IV. The values for white tracts and peripheral nervous tissue are so low as to be of doubtful significance. Transaminase activity in different

[1] All enzyme activities are expressed in mmoles of product formed per kg. of tissue and per hour in terms of dry weight of tissue and at 38° unless otherwise noted.

TABLE III

Distribution of glutamic-γ-aminobutyric transaminase in gray matter of spinal cord

	Lumbar	Thoracic	Cervical
	mmoles/kg./hour		
A*	197 ± 13	223 ± 8	261 ± 7
B	202 ± 9		
C	246 ± 20		
D	315 ± 23	240 ± 13	287 ± 15

* The letters refer to Fig. 3.

TABLE IV

Glutamic-γ-aminobutyric transaminase activity in peripheral elements of nervous system

	mmoles/kg./hour
Dorsal roots	0
Ventral roots	0
Adrenal cortex	0
Dorsal ganglion	8
Adrenal medulla	15
Superior cervical ganglion	20

areas of gray matter varies over a 35-fold range from 9 mmoles per kg. per hour in layers 5 and 6 of occipital cortex to 315 mmoles per kg. per hour in the dorsal horn of lumbar spinal cord. Moreover, within adjacent gray areas of the same morphological structure, 6-fold variations have been found (*e.g.* occipital cortex). The higher levels of activity are found in subcortical structures with the highest values occurring in the midbrain, brain stem, and spinal cord. However, there are gray areas, such as the red nucleus and substantia nigra in the midbrain, which do not fit into this rostral-caudal gradient of activity.

DISCUSSION

Although only indirect evidence has been advanced to support neurohumoral (14, 15) or homeostatic (16) functions for γ-aminobutyrate, the presence and rapid turnover of γ-aminobutyrate in mammalian brain are conclusively established facts. The net result (Reaction 4) of the reactions catalyzed by glutamic decarboxylase, glutamic-γ-aminobutyric transaminase and semialdehyde dehydrogenase reactions (Reactions 1 to 3), permits glutamate and γ-aminobutyrate to be considered as intermediates in the oxidation of α-ketoglutarate (6).

$$\text{Glutamate} \rightarrow \gamma\text{-amino butyrate} + CO_2 \quad (1)$$

$$\gamma\text{-Aminobutyrate} + \alpha\text{-ketoglutarate} \rightarrow \text{glutamate} + \text{succinic semialdehyde} \quad (2)$$

$$\text{Succinic semialdehyde} + \text{DPN} \rightarrow \text{succinate} + \text{DPNH} \quad (3)$$

$$\alpha\text{-Ketoglutarate} + \text{DPN} \rightarrow \text{succinate} + CO_2 + \text{DPNH} \quad (4)$$

The *in vivo* rate of oxygen metabolism in human brain is about 90 mmoles per kg. per hour (17). Since the respiratory quotient of brain is nearly 1.0, the oxidative decarboxylation of 30 mmoles per kg. per hour of α-ketoglutarate is required. Thus, on a dry weight basis, any reaction in this pathway must have a total capacity of at least 150 mmoles per kg. per hour. The highest value we have found in any region of monkey brain is approximately twice this figure.

Most enzymes of the glycolytic pathway and the tricarboxylic acid cycle exhibit activities *in vitro* which are one or two orders of magnitude greater than the activity required by the metabolic rate *in vivo* (18). Values which have been reported for pyruvic oxidase (19) and condensing enzyme (19) *in vitro* are much nearer the calculated minimum.

It is apparent from the observed enzymatic activity that the glutamic-γ-aminobutyric transaminase cannot function in the principal pathway of carbohydrate metabolism in brain as a whole. The possibility remains that major importance may be attached to this pathway as an energy source at particular cytological loci. Although very little quantitative histological information is available for correlation with the enzyme data, the following deductive correlations may be made. Any histological elements correlated with glutamic-γ-aminobutyric transaminase activity must be virtually absent from white matter. The possibilities are neuronal cell bodies, synaptic processes, Golgi type II neurons, and protoplasmic astrocytes. Neuronal cell bodies, synapses, and Golgi type II cells are all found in profusion in the cerebral cortex where transaminase activity is rather low. Golgi type II cells are few or absent from areas such as dentate nucleus and inferior olivary nucleus where glutamic-γ-aminobutyric transaminase activity is high. Little basis exists for evaluation of the distribution of protoplasmic astrocytes since methods for demonstrating them are highly capricious by quantitative standards. The granular layer of cerebellar cortex contains many protoplasmic astrocytes but has only moderate glutamic-γ-aminobutyric transaminase activity.

All of the preceding deductions assume homogeneity of the morphological classifications. Since none of the classifications account for the observed differences in the enzyme distribution, a biochemical heterogeneity must exist within one or more of these superficially similar morphological groupings. Further examination of these possibilities must await methods which are sufficiently sensitive to examine the glutamic-γ-aminobutyric transaminase activity of the primary neurons of areas such as the dentate nucleus, inferior olive, and the colliculi.

SUMMARY

Glutamic-γ-aminobutyric transaminase activity has been measured in a quantitative histochemical survey of the nervous system of the monkey. The enzyme assay procedure was based upon the fluorometric measurement of the condensation product of succinic semialdehyde with 3,5-diaminobenzoic acid. Conditions for the assay are described which permit measurement of activity on 3 to 15 μg. of lyophilized brain tissue. The enzyme was found to be largely confined to the gray matter of the central nervous system and, in general, to exhibit progressively increasing activity in subcortical and lower structures.

REFERENCES

1. TSUKADA, Y., NAGATA, Y., AND TAKAGAKI, G., *Proc. Japan Acad.*, **33**, 510 (1957).
2. MCKHANN, G. M., AND TOWER, D. B., *Am. J. Physiol.*, in press.
3. BESSMAN, S. P., ROSSEN, J., AND LAYNE, E. C., *J. Biol. Chem.*, **201**, 385 (1953).
4. ROBERTS, E., AND FRANKEL, S., *J. Biol. Chem.*, **187**, 55 (1950).
5. ALBERS, R. W., AND SALVADOR, R. A., *Science*, **128**, 359 (1958).

6. Roberts, E., in S. R. Korey and J. I. Nurnberger (Editors), *Progress in neurobiology, Vol. I*, Paul B. Hoeber, Inc., New York, 1956, p. 11.
7. Carrière, E., *Ann. Chim. Rome*, **17**, 38 (1922).
8. Lowry, O. H., *J. Histochem. Cytochem.*, **1**, 420 (1953).
9. Velluz, L., Amiard, G., and Pesez, M., *Bull. Soc. chim. France*, **15**, 678 (1948).
10. Kissane, J. M., and Robins, E., *J. Biol. Chem.*, **233**, 184 (1958).
11. Scott, E. M., and Jakoby, W. B., *Science*, **128** (1958).
12. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L., and Farr, A. L., *J. Biol. Chem.*, **207**, 1 (1954).
13. Albers, R. W., and Salvador, R. A., *Federation Proc.*, **17**, 2 (1958).
14. Bazemore, A. W., Elliott, K. A. C., Florey, E., *J. Neurochem.*, **1**, 334 (1957).
15. Florey, E., and McLennan, H., *J. Physiol. London*, **130**, 446 (1955).
16. Killam, K. F., *J. Pharmacol. Exptl. Therap.*, **119**, 263 (1957).
17. Kety, S. S., *Methods in Med. Research*, **1**, 204 (1948).
18. McIlwain, H., *Biochemistry and the central nervous system*, J. and A. Churchill, Ltd., London, 1955.
19. Coxon, R. V., and Peters, R. A., *Biochem. J.*, **46**, 300 (1950).

The Distribution of Glutamic Decarboxylase in the Nervous System of the Rhesus Monkey

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A distinctive pattern of glutamic-γ-aminobutyric transaminase activity in the nervous system has been demonstrated in the preceding study (1). γ-Aminobutyric acid occurs in uniquely high concentrations in brain tissue, and arises from the decarboxylation of glutamic acid (2). In the present experiments, glutamic decarboxylase activity has been determined in microgram quantities of nervous tissue by a microradiometric method. The levels of glutamic decarboxylase and glutamic-γ-aminobutyric transaminase have been measured in comparable regions of the nervous system.

EXPERIMENTAL

Methods and Material

The method of Passmann *et al.* (3) for absorbing and counting $C^{14}O_2$ by liquid scintillation spectrometry was modified for use with substrates of high specific activity to detect as little as 10^{-11} moles of CO_2. The technique has been designed to permit the analysis of large numbers of samples with a minimum of manipulation.

Apparatus and Reagents—In addition to the usual apparatus for quantitative histochemistry (4, 5), special reaction tubes, counting tubes, and a micromanipulator were used for handling the radioactive materials. The reaction and counting tubes were constructed of Pyrex tubing according to the dimensions indicated in the legend to Fig. 1. The polyethylene sleeves were cut from size 360 polyethylene tubing. A Brinkman MP-V micromanipulator was used to hold the reaction tube and a Brinkman RP-III micromanipulator was employed for holding the pipet in the substrate loading operation. The liquid scintillation counter was a Packard Tri-Carb model A.

The incubation medium contained 0.1 M L-glutamate-U-C^{14} (2.8 μc. per μmole), and was purchased from Nuclear-Chicago Corporation. The medium was buffered with 0.1 M potassium phosphate at pH 6.8 and contained 5×10^{-4} M pyridoxal phosphate (California Biochemical Corporation). The free base of *p* - (diisobutylcresoxyethoxyethyl)dibenzylammonium chloride (Hyamine) was prepared according to the method of Passmann *et al.* (3). The counting fluid contained 0.05 M Hyamine, 1 per cent (volume for volume) methanol, 0.001 per cent (weight per volume) β-bis[2-(phenyloxazolyl)]-benzene and 0.4 per cent weight per volume 2,5-diphenyloxazole in toluene.

Procedure—Frozen-dried histological sections were dissected from the same material prepared for the study of the distribution of glutamic-γ-aminobutyric transaminase (1). Samples weighing between 3 to 15 μg. were carefully dissected and weighed on a quartz fiber balance. The samples were placed in the reaction tubes, capped with Parafilm, and stored at −20° until analysis.

During the addition of reagents, the rack of samples was kept in an ice bath. Two μl. of a 0.3 per cent solution of bovine serum albumin were added to each tube. This was followed by the addition of 2.5 μl. of buffer-substrate solution from a straight tipped pipet. The latter operation was performed with both reaction tube and pipet controlled by micromanipulators and observed with a stereomicroscope to insure that the addition was made quantitatively into the albumin without contaminating the wall of the reaction tube.

After the addition of substrate, a 10- to 15-μl. droplet of 5 N H_2SO_4 was placed across the lumen of the reaction tube about one-third down from the top (Fig. 1*A*). The tubes were then capped with Parafilm and incubated in an oven at 38° for 2 hours. At the end of this period, 50 μl. of counting fluid were added to each counting tube. The Parafilm cap was removed from the incubation tube, and the second tube containing the counting mixture was sealed to the reaction tube with a polyethylene sleeve. The sleeve was bent so that the counting and reaction tubes were nearly side by side and then the acid droplet was shaken down into the reaction mixture and mixed with it. The tubes were placed horizontally in the extended position at 38° (Fig. 1*B*) for 2 hours to allow the CO_2 to diffuse quantitatively into the counting fluid.

At the end of the diffusion period, the tubes were secured in the side-by-side position with a rubber band (Fig. 1*C*). A black paper sleeve was placed around the reaction tube to avoid possible error which might have arisen from contamination of the reaction tube with phosphor. The base of the counting tube was then inserted into an adapter made from a rubber stopper and placed in the counting well of the scintillation counter.

Blank values were obtained by carrying reaction mixtures without tissue through the procedure. Control tissue samples for checking one day's analyses with those of another were obtained by preparing reaction tubes with equal amounts of whole brain homogenate and lyophilizing these homogenates in the reaction tubes. Triplicate control determinations were made with each set of analyses. The specific activity of the evolved $C^{14}O_2$ was calculated from that of the substrate by directly counting a standard dilution of the substrate.

Protein was determined by the method of Lowry *et al.* (6).

Comment on Method—The glutamic acid-C^{14} is subject to slow radio-decomposition to a product that is somewhat volatile. This decomposition product, which is not retained on Dowex

50(H+), accounts for as much as 7 per cent of the total radioactivity in some vials. It was found that blank "$C^{14}O_2$" values from the incubation media alone were slightly greater than those obtained in the presence of heat-inactivated brain homogenate. Bovine serum albumin also exerted this suppressing effect on blank values and was therefore included in the incubation medium.

The rate of diffusion of $C^{14}O_2$ in the microtubes was checked with use of $NaHC^{14}O_3$ in model experiments. Diffusion was nearly complete in 30 minutes at 38°.

Glutamic decarboxylase activity decreases with increasing incubation times in a reproducible manner (Fig. 2). All results are corrected to the activity at 30 minutes of incubation.

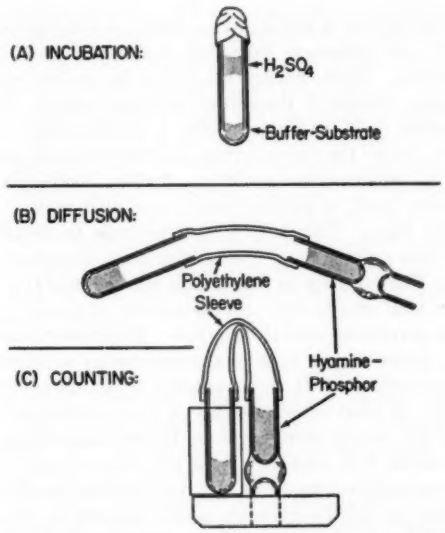

FIG. 1. Technique for measuring enzymatically produced $C^{14}O_2$. (*A*) The reaction tube is made of Pyrex glass tubing, 4 × 35 mm. (*B*) The counting tube is connected to the reaction tube by a short length of polyethylene tubing during the diffusion period. The counting tube consists of a 4 × 45 mm. length of Pyrex glass tubing which is sealed about 20 mm. from the lower end. (*C*) The tubes are shown in position for counting.

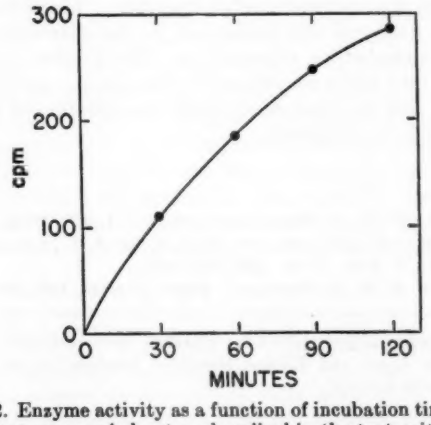

FIG. 2. Enzyme activity as a function of incubation time. The experiment was carried out as described in the text, with the exception that the samples consisted of a 1:30 homogenate of monkey brain in 0.3 per cent bovine albumin.

Direct counting in the volume (50 μl.) of phosphor in the microtubes was compared with counting in the standard 15-ml. glass vials with palmitic acid-C^{14}. In contrast to counting in the standard vials, the phenyloxazolylbenzene was found markedly to increase the efficiency in the microtubes. Approximately the same counting efficiency, with no significant increase in background, was achieved with the microtubes at a slightly higher phototube voltage than was used with the 15-ml. vials.

RESULTS

The decarboxylase activity was examined in homogenates of grossly dissected areas of the monkey central nervous system (Table I). The highest activity was found in the hypothalamus and mesencephalon. The spinal cord and brain stem were markedly less active. None of the white tracts of the spinal

TABLE I

Distribution of glutamic decarboxylase in gross regions of central nervous system

	Mmoles/Kg./hour (protein)*
Whole brain	44
Midbrain	100
Hypothalamus	96
Thalamus	85
Occipital cortex	84
Cerebellum	79
Frontal cortex	70
Motor cortex	62
Rhinencephalon	60
Pons and medulla	34
Lumbar spinal cord	15

* The figures are average values obtained from the analysis of triplicate aliquots of homogenates prepared from a single monkey brain. Values are given as mmoles of glutamic acid decarboxylated per kg. of protein per hour.

TABLE II

Glutamic decarboxylase in monkey spinal cord

Enzyme activity is reported in mmoles per kg. dry weight and per hour at 38°. The standard error of the mean is calculated from 3 to 6 samples from a single animal. The regions of the spinal gray matter, *A*, *B*, *C*, and *D*, are indicated in Fig. 3 of the preceding paper (1).

	Mmoles/kg./hour	T/D*
Lumbar gray matter		
A	6.8 ± 3.1	29
B	11.5 ± 1.5	18
C	16.9 ± 1.5	14
D	25.6 ± 5.9	12
Substantia gelatinosa	19.9 ± 1.7	
Nucleus proprius dorsalis	20.2 ± 6.1	
Lateral pyramidal tract	0	
Ventral root	2.5 ± 1.0	
Dorsal root	1.6 ± 1.2	
Dorsal ganglion	1.6 ± 1.6	
Superior cervical ganglion	1.2 ± 1.0	
Adrenal medulla	1.8 ± 0.6	

* The observed ratio of glutamic-γ-aminobutyric transaminase activity to glutamic decarboxylase activity.

TABLE III

Glutamic decarboxylase in cortical structures

	Mmoles/kg./hour	T/D*
Cerebellum		
Molecular layer	53.9 ± 11.0	2.8
Granular layer	46.4 ± 2.1	1.6
Hippocampus		
Alveus	17.1 ± 6.5	
Pyramidalis	32.9 ± 4.3	2.1
Radiata	25.0 ± 6.7	2.6
Molecularis	35.5 ± 2.2	2.6
Occipital cortex		
Layers 1–2	25.0†	
Layers 3–4a	57.7 ± 5.0	
Layers 4b–5	19.4 ± 4.9	
Layer 6	13.3 ± 3.8	
White	0	

* See Table II.

† Only two samples were analyzed.

TABLE IV

Glutamic decarboxylase in subcortical structures

	Mmoles/kg./hour	T/D*
Substantia nigra (z. reticularis)	68.4 ± 14.1	1.4
Substantia nigra (z. compacta)	65.8 ± 12.4	
Reticular formation	32.4 ± 5.3	3.5
Supra optic nucleus	23.3 ± 5.2	
Red nucleus (parvocellular)	20.1 ± 4.1	3.1
Red nucleus (magnocellular)	16.1 ± 4.8	
Midbrain central gray (lateral)	15.6 ± 0.3	6.8
Anterior thalamic nucleus	14.8 ± 2.1	10
Globus pallidus	14.3 ± 7	3.6
Midbrain central gray (medial)	10.9 ± 2.8	7.3
Lateral thalamic nucleus	8.3 ± 2.3	6.4
Abducens nucleus	7.6 ± 0.9	27
Inferior olivary nucleus	5.7 ± 0.2	52
Superior colliculus (external and middle gray)	5.3 ± 0.8	52
Posterior pituitary	1.5 ± 0.7	
Pineal gland	0	

* See Table II.

cord which were examined, the spinal nerve roots, nor the dorsal ganglia possessed appreciable activity (Table II). In the gray matter of the spinal cord, a marked dorsoventral gradient occurred, the enzymatic activity of the dorsal horn being among the higher values found in the central nervous system.

In the cerebellum and hippocampus, the different histological subdivisions exhibited rather similar activities. However, in the visual cortex, a definite maximum is apparent in layers 2 and 3 (Table III).

Gray matter throughout the brain exhibited a wide range of decarboxylase activities; the highest value of any region was found in the substantia nigra (Table IV). The superior cervical ganglion and adrenal medulla had very low activity.

DISCUSSION

The distribution of glutamic decarboxylase exhibits both similarities to and differences from that of glutamic-γ-aminobutyric transaminase. Both enzymes are seen to be confined essentially to the gray matter of the central nervous system. In spinal gray matter, both enzymes exhibit a ventral-dorsal gradient. However, unlike the transaminase, no rostral-caudal gradient is evident in decarboxylase activity. Rather, distinctively high values were found in cerebral cortex, cerebellar cortex,[1] and the substantia nigra. The ratio of transaminase to decarboxylase activity was greater than 1 in all the areas examined (Tables II–IV). The variation in this ratio is marked, and it is possible that the local steady-state concentrations of γ-aminobutyrate might be correlated with these ratios. This observation is particularly interesting in view of the association of altered levels of γ-aminobutyrate with the production of convulsion by hydrazides (7). If local concentrations of γ-aminobutyrate are the result of the steady state governed by transamination and decarboxylation, it is probable, from the values found, that glutamic decarboxylase would be the rate-limiting reaction. Substantiation of this hypothesis would support a homeostatic function for γ-aminobutyrate in cerebral function rather than a neurohumoral function in which local concentration would be regulated by storage and release mechanisms.

SUMMARY

A quantitative histochemical survey of glutamic decarboxylase activity in the nervous system of the monkey has been made by means of a microradiometric procedure. The histochemical data are compared with similar data for the distribution of glutamic-γ-aminobutyric transaminase. The possible relationship between the relative activities of the transaminase and the decarboxylase and the local steady-state concentration of γ-aminobutyric acid is considered.

REFERENCES

1. SALVADOR, R. A., AND ALBERS, R. W., *J. Biol. Chem.*, **234**, 922 (1959).
2. ROBERTS, E., in S. R. KOREY AND J. I. NURNBERGER (Editors), *Progress in neurobiology, Vol. I*, Paul B. Hoeber, Inc., New York, 1956.
3. PASSMANN, J. M., RADIN, N. S., AND COOPER, J. A. D., *Anal. Chem.*, **28**, 484 (1956).
4. LOWRY, O. H., ROBERTS, N. R., LEINER, K. Y., WU, M.-L., AND FARR, A. L., *J. Biol. Chem.*, **207**, 1 (1954).
5. LOWRY, O. H., *J. Histochem. Cytochem.*, **1**, 420 (1953).
6. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
7. KILLAM, K. F., *J. Pharmacol. Exptl. Therap.*, **119**, 263 (1957).

[1] Somewhat higher values for glutamic decarboxylase were observed by Lowe and Robins (personal communication) with a fluorometric method.

Effect of Hyperthyroidism on Liver Pyridine Nucleotide Synthesis*

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The concentration of pyridine nucleotides in the liver of the hyperthyroid rat is low (1–4). Whether this is a result of decreased synthesis, increased destruction, or both, is not known. Several groups of investigators have shown that when the normal rat or the normal mouse is given a single dose of nicotinamide the concentration of pyridine nucleotides in the liver rises rapidly for a few hours and then falls to within the normal range within 24 hours (5–7). This procedure which should give a measure of the capacity of the rat to synthesize liver pyridine nucleotides, has been used in the present investigation to study the rate and extent of synthesis of pyridine nucleotides in the liver of the hyperthyroid rat, the hyperthyroid mouse and the rat treated with 2,4-dinitrophenol.

EXPERIMENTAL

Male weanling white rats,[1] weighing 40 to 50 gm., and male weanling white mice,[2] weighing 9 to 11 gm., were used in these experiments. All animals were housed in suspended, screen-bottom cages and were fed *ad libitum*.

The diets used in these studies contained generous amounts of all known essential nutrients. The diets contained 26.3 per cent of casein, 5 per cent of corn oil, 5 per cent of salts, a complete vitamin mixture, and sucrose. The exact compositions of the diets have been described (8).

Rats were made hyperthyroid by including 0.4 per cent of iodinated casein[3] in their diets. After the rats had been fed for 19 to 20 days on the experimental diets, pyridine nucleotide synthesis was studied *in vivo*. All mice were fed the casein basal diet for 4 days to allow them to adjust to a purified diet; then, they were fed either the casein basal diet or the casein diet containing 0.4 per cent of iodinated casein for 14 additional days, after which pyridine nucleotide synthesis was studied *in vivo*.

For the experiments on the effect of dinitrophenol, rats were fed the casein basal diet and 2,4-dinitrophenol was administered by intraperitoneal injection as follows. Doses of 2 mg. per 100 gm. of body weight were given twice daily for 8 days; then, doses of 3 mg. per 100 gm. of body weight were given twice daily for the next 9 days. Although Tainter and Cutting (9) found that the LD_{50} for 2,4-dinitrophenol in rats was 2.5 mg. per 100 gm. of body weight, very few of the rats injected with dinitrophenol died during the present experiments.

Pyridine nucleotide synthesis was studied as follows. A single dose of 100 mg. of nicotinamide per 100 gm. of body weight was injected intraperitoneally. An equivalent volume of 0.9 per cent sodium chloride solution was injected into control animals. Food cups were removed from the cages after the animals had been injected; however, results obtained in a series of experiments not reported, in which the animals were allowed to eat after the nicotinamide or sodium chloride injection, indicated that this procedure was without effect on pyridine nucleotide synthesis. In the dinitrophenol studies, either nicotinamide or sodium chloride solution was injected 2 hours after the final injection of 2,4-dinitrophenol on the 17th day. Liver pyridine nucleotide concentrations were determined by the method of Feigelson *et al.* (10). This method of analysis does not distinguish between DPN and TPN but rather measures their sum.

RESULTS

The effect of hyperthyroidism on liver pyridine nucleotide accumulation following the administration of nicotinamide is shown in Fig. 1. Groups of rats were killed at the times indicated after a single injection of either nicotinamide or sodium chloride solution, and the concentration of pyridine nucleotides in each liver was determined. Each point on the curves represents the mean of the values for 5 to 8 rats.

Average initial concentrations of pyridine nucleotides in the liver of the hyperthyroid and the normal rat were 760 and 960 μg. per gm. of fresh liver, respectively. The concentration rose rapidly in both the normal and the hyperthyroid rat following the injection of nicotinamide and reached a maximum in approximately 4 hours. Although the concentration of pyridine nucleotides in the liver of the hyperthyroid rat was initially lower, the rate of synthesis during the first 2 hours following the injection was similar to that observed in the normal rat. After 2 hours there was little additional synthesis of pyridine nucleotides in the liver of the hyperthyroid rat, but in the normal rat the rapid rate of synthesis continued until the maximal pyridine nucleotide concentration was reached in 4 hours. At this time there was a marked difference between the liver pyridine nucleotide concentration of the hyperthyroid rat and that of the normal rat.

After 4 hours, there was a gradual decrease in pyridine nucleotide concentration, the rate of decrease being similar in both the hyperthyroid and the normal rat. The injection of sodium

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[1] Obtained from the Holtzman Company, Madison, Wisconsin.

[2] Obtained from the Dan Rolfsmeyer Company, Madison, Wisconsin.

[3] Protamone, Cerophyl Laboratories, Kansas City, Missouri.

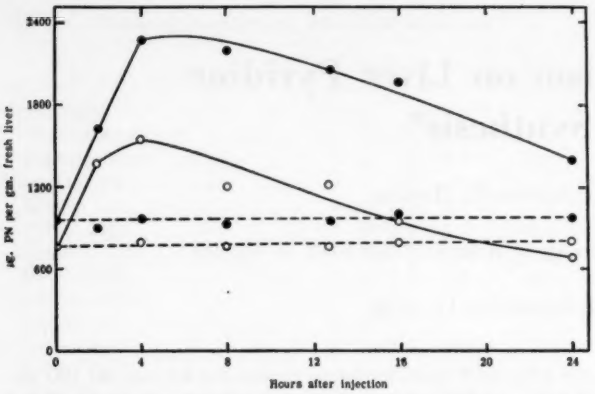

FIG. 1. Liver pyridine nucleotide concentration in hyperthyroid and normal rats after a single injection of nicotinamide or sodium chloride. Rats were injected intraperitoneally with 100 mg. of nicotinamide per 100 gm. of body weight or an equivalent volume of sodium chloride solution. At each of the times indicated, 5 to 8 rats were killed and their liver pyridine nucleotide concentrations were determined. ●——●, normal rats injected with nicotinamide; ○——○, hyperthyroid rats injected with nicotinamide; ●-----●, normal rats injected with sodium chloride; ○-----○, hyperthyroid rats injected with sodium chloride. PN, pyridine nucleotide.

TABLE I

Liver pyridine nucleotide concentration of hyperthyroid mice 12 hours after nicotinamide or sodium chloride administration

	Group	Treatment	Mice per group	Total pyridine nucleotide per gm. fresh liver
				μg.
1	Normal mice.....	Sodium chloride injected	9	699 ± 16*
	Normal mice.....	Nicotinamide injected	10	3260 ± 194
2	Hyperthyroid mice....	Sodium chloride injected	5	633 ± 18
	Hyperthyroid mice....	Nicotinamide injected	4	1376 ± 192

* Mean ± standard error of the mean.

chloride solution caused no change in the liver pyridine nucleotide concentration of either the hyperthyroid or the normal rat.

The effect of an injection of nicotinamide on the concentration of pyridine nucleotides in the liver is much greater in the mouse than in the rat (7). Since, in the rat, hyperthyroidism markedly decreased liver pyridine nucleotide accumulation 4 to 16 hours after an injection of nicotinamide, the effect of hyperthyroidism on liver pyridine nucleotide synthesis in the mouse was also determined. Normal and hyperthyroid mice were injected with either 100 mg. of nicotinamide per 100 gm. of body weight or with an equivalent volume of sodium chloride solution. They were killed 12 hours later, and their liver pyridine nucleotide concentrations were determined.

The results of this experiment (Table I) show that the concentration of liver pyridine nucleotides was much lower in the mouse (699 μg. per gm. of fresh liver) than in the rat (960 μg. per gm. of fresh liver), although both species of animals received the same diet. Hyperthyroidism caused the concentration of pyridine nucleotides in the liver of the mouse to fall, but not to the same extent, either on an absolute or on a percentage basis, as it did in the rat. Also, the accumulation of liver pyridine nucleotides was low in the hyperthyroid mouse 12 hours after an injection of nicotinamide. It thus seems quite likely that the effect of hyperthyroidism on pyridine nucleotide metabolism is similar in the rat and in the mouse.

Thyroxine causes uncoupling of oxidative phosphorylation, both *in vitro* and *in vivo* (11, 12). This may be an indirect effect (13), but since ATP is required in the biosynthesis of DPN from nicotinamide (14, 15), a decrease in the amount of ATP available as a result of uncoupling could cause a decrease in the rate of pyridine nucleotide synthesis. Of a number of other substances which cause uncoupling of oxidative phosphorylation *in vitro*, 2,4-dinitrophenol is perhaps the best known. Dianzani and Scuro (16) have reported that 2,4-dinitrophenol will cause uncoupling *in vivo* but Parker (17) could not show such an effect. In order to determine whether 2,4-dinitrophenol might also inhibit liver pyridine nucleotide synthesis, the extent of pyridine nucleotide synthesis in rats which had been injected with 2,4-dinitrophenol was studied. Severe fever and an increased metabolic rate are seen shortly after the administration of 2,4-dinitrophenol but the rat appears quite normal 6 to 8 hours later (18); therefore, nicotinamide was injected 2 hours after the final injection of 2,4-dinitrophenol in the first experiment.

The results of this experiment are given in Fig. 2. Each point on the curves represents the mean of the liver pyridine nucleotide values for 5 to 10 rats killed at the times indicated after a single injection of nicotinamide or sodium chloride solution. Treatment of the rat with 2,4-dinitrophenol did not affect the "resting" concentration of pyridine nucleotides in the liver (960 μg. per gm. of fresh liver). Also, 4 hours after these rats had been given a single nicotinamide injection the liver pyridine nucleotide concentration was 2260 μg. per gm. of fresh liver. Thus, since both the rate of synthesis and the magnitude of the pyridine

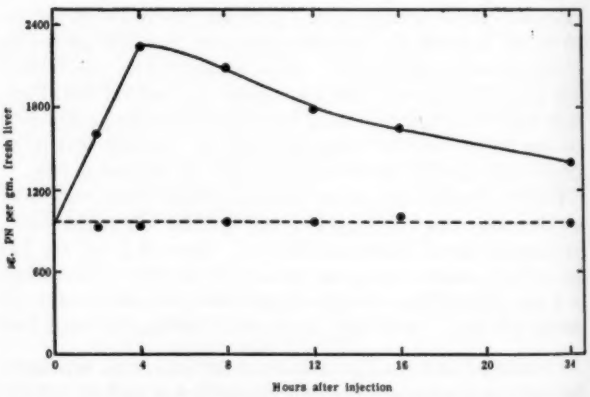

FIG. 2. Liver pyridine nucleotide concentration in dinitrophenol-treated rats after a single injection of nicotinamide or sodium chloride. Rats were injected intraperitoneally with 100 mg. of nicotinamide per 100 gm. of body weight or an equivalent volume of sodium chloride solution. At each of the times indicated, 5 to 10 rats were killed and their liver pyridine nucleotide concentrations were determined. ●——●, injected with nicotinamide; ●-----●injected with sodium chloride. PN, pyridine nucleotide.

nucleotide accumulation were the same as in the normal rat (see Fig. 1), synthesis was apparently unaffected by treatment of the animals with 2,4-dinitrophenol.

In another experiment, two groups of rats were treated as follows. One group was given two injections of 2,4-dinitrophenol, 6 hours and 2 hours before the nicotinamide injection; the other group was injected with 2,4-dinitrophenol and nicotinamide at the same time. All rats were killed 4 hours after the injection of nicotinamide and the concentration of pyridine nucleotides in their livers was determined. Neither of these treatments affected the rate nor the magnitude of the pyridine nucleotide accumulation. Likewise, when weanling rats were fed 0.1 per cent of 2,4-dinitrophenol in the diet for 20 days, pyridine nucleotide synthesis after an injection of nicotinamide was the same as in the normal rat.

After 4 hours, the rate of decrease in the liver pyridine nucleotide concentration of rats treated with 2,4-dinitrophenol seemed to be a little greater than that observed in the normal rat. However, the experiments with normal rats and those with rats treated with 2,4-dinitrophenol were not done at the same time and the standard errors of the means were quite high; since both 4 and 24 hours after the nicotinamide injection the liver pyridine nucleotide concentrations were the same as those of the normal rat (see Fig. 1), it appears justifiable to conclude that treatment of the rat with 2,4-dinitrophenol under the conditions of this experiment had no significant effect on pyridine nucleotide metabolism.

DISCUSSION

It is evident that an injection of nicotinamide causes a much smaller accumulation of pyridine nucleotides in the liver of the hyperthyroid animal than in that of the normal animal. This could be the result of (*a*) increased destruction of pyridine nucleotides, or (*b*) decreased synthesis of pyridine nucleotides, due either to a decreased enzyme concentration or to a lack of precursor.

The rates of destruction of pyridine nucleotides in the livers of both the hyperthyroid and the normal rat appeared to be similar because, between 4 and 24 hours after an injection of nicotinamide, the newly synthesized pyridine nucleotides disappeared from the liver at nearly the same rate in both groups. If the rate of destruction of pyridine nucleotides were greater in the hyperthyroid rat, the slope of the curve for pyridine nucleotide disappearance should have been much steeper than that for the normal rat. Therefore, the smaller accumulation of pyridine nucleotides in the liver after an injection of nicotinamide into the hyperthyroid rat would not seem to result from an increase in the rate of pyridine nucleotide destruction.

The lower accumulation of pyridine nucleotides does not appear to be caused by a defect in the enzyme system responsible for the synthesis of these coenzymes from their immediate precursors because the rates of synthesis in the liver of the normal rat and in that of the hyperthyroid rat were similar for the first 2 hours. Also, since Maley and Lardy (2) found that the rates of DPN synthesis from NMN and ATP *in vitro* were the same in liver nuclei preparations from the normal and the hyperthyroid rat, this enzyme would appear to be present in normal concentrations in hyperthyroid rat liver.

The shape of the curve for pyridine nucleotide formation suggests that the synthesis of these coenzymes occurs at a normal rate immediately after nicotinamide injection, then falls off rapidly because of the depletion of some component that is required for this synthesis. The decreased accumulation of pyridine nucleotides could be due to an inadequate supply of ATP, of ribose, of adenine, or of NMN, all of which are needed for DPN synthesis when nicotinamide is supplied in excess. The concentrations of ATP and creatine phosphate in heart muscle from the hyperthyroid rat are considerably lower than is the case in the normal rat (19), but 2,4-dinitrophenol administration failed to affect liver pyridine nucleotide metabolism *in vivo*. If, as was concluded by Parker (17), 2,4-dinitrophenol does not cause uncoupling of oxidative phosphorylation *in vivo*, no depression of ATP synthesis should occur. Regardless of the nature of the defect causing a depression of pyridine nucleotide synthesis in the hyperthyroid rat, the condition cannot be duplicated by treatment with 2,4-dinitrophenol.

SUMMARY

1. The values for pyridine nucleotide concentrations in the livers of hyperthyroid rats 4 to 24 hours following an injection of nicotinamide, were well below those for similarly treated euthyroid rats. The liver pyridine nucleotide concentration of hyperthyroid mice was also much lower than that of euthyroid mice 12 hours after an injection of nicotinamide.

2. Treatment of rats with dinitrophenol did not affect their ability to synthesize liver pyridine nucleotides after an injection of nicotinamide.

REFERENCES

1. Katzenelbogen, E., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **141**, 611 (1941).
2. Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, **215**, 377 (1955).
3. Glock, G. E., and McLean, P., *Biochem. J.*, **61**, 397 (1955).
4. Bosch, A. J., Ph.D. Thesis, University of Wisconsin, 1958.
5. Feigelson, P., Williams, J. N., Jr., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **78**, 34 (1951).
6. Kaplan, N. O., Goldin, A., Humphreys, S. R., Ciotti, M. M., and Stolzenbach, F. E., *J. Biol. Chem.*, **219**, 287 (1956).
7. Narurkar, M. V., Kumta, U. S., and Sahasrabudhe, M. B., *Brit. J. Cancer*, **11**, 482 (1957).
8. Boldt, R. E., Harper, A. E., and Elvehjem, C. A., *Arch. Biochem. Biophys.*, **74**, 437 (1958).
9. Tainter, M. L., and Cutting, W. C., *J. Pharmacol. Exptl. Therap.*, **49**, 187 (1933).
10. Feigelson, P., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **185**, 741 (1950).
11. Martius, C., and Hess, B., *Arch. Biochem. Biophys.*, **33**, 486 (1951).
12. Lardy, H. A., and Feldott, G., *Ann. N. Y. Acad. Sci.*, **54**, 636 (1951).
13. Tapley, D. F., and Cooper, C., *J. Biol. Chem.*, **222**, 341 (1956).
14. Kornberg, A., *J. Biol. Chem.*, **182**, 779 (1950).
15. Rowen, J. W., and Kornberg, A., *J. Biol. Chem.*, **193**, 497 (1951).
16. Dianzani, M. U., and Scuro, S., *Biochem. J.*, **62**, 205 (1956).
17. Parker, V. H., *Nature*, **178**, 261 (1956).
18. Tainter, M. L., and Cutting, W. C., *J. Pharmacol. Exptl. Therap.*, **48**, 410 (1933).
19. Mascitelli-Coriandoli, E., and Boldrini, R., *Nature*, **179**, 1196 (1957).

Soluble γ-Aminobutyric-Glutamic Transaminase from *Pseudomonas fluorescens*

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The presence of relatively high concentrations of γ-aminobutyric acid in mammalian brain (1, 2) led to the discovery of a reaction utilizing this amino acid by what appeared to be transamination with α-ketoglutaric acid (3, 4). Studies with brain preparations (3, 4) were, however, handicapped by the particulate nature of the system catalyzing the reaction. Although active, soluble extracts were obtained from microorganisms (5–7), these were not further purified.

Recent studies have implicated γ-aminobutyric-glutamic transaminase (8) and succinic semialdehyde dehydrogenase (8, 9) as the successive, terminal steps in the conversion of pyrrolidine to succinic acid by a strain of *Pseudomonas fluorescens*. Preliminary evidence suggested (8) that pyrrolidine was metabolized by way of the following successive intermediates: Δ^1-pyrroline,[1] γ-aminobutyric acid, succinic semialdehyde, and succinic acid.

The present report summarizes the data on the purification and properties of γ-aminobutyric-glutamic transaminase (Reaction 1) and presents a kinetic analysis of the reaction which it catalyzes.

$$\gamma\text{-Aminobutyrate} + \alpha\text{-ketoglutarate} \rightleftarrows \text{succinic semialdehyde} + \text{glutamate} \quad (1)$$

Preparation of Enzyme

Assay—Enzyme activity was determined by coupling Reaction 1 with an excess of succinic semialdehyde dehydrogenase (Reaction 2) so that the formation of reduced pyridine nucleotide was a function of transaminase activity.

$$\text{Succinic semialdehyde} + \text{DPN}^+ \text{ (or TPN}^+) + H_2O \rightarrow \text{succinic acid} + \text{DPNH (or TPNH)} + H^+. \quad (2)$$

The reaction was followed spectrophotometrically at 340 mμ in an incubation system which included the following (in μmoles) in a total volume of 1.0 ml.: potassium pyrophosphate at pH 8.1, 100; α-ketoglutarate, 1; γ-aminobutyrate, 1; pyridine nucleotide, 0.5; and excess of succinic semialdehyde dehydrogenase sufficient to catalyze the oxidation of 0.1 μmole of succinic semialdehyde per minute. Two such dehydrogenase preparations, both of which catalyze experimentally irreversible reactions, were used. Most of the work was performed with a fraction isolated from a preparation of the DPN-linked yeast aldehyde dehydrogenase described by Black (10).[2] Alternatively, the substrate specific, succinic semialdehyde dehydrogenase from *P. fluorescens* (9), utilizing either TPN or DPN, was employed. Although the pH of the standard incubation system was not optimal, considerable endogenous activity, *i.e.* reduction of pyridine nucleotide, found with the cruder fractions at higher pH values was minimized. Activity was followed at 1-minute intervals for 5 minutes. Transaminase activity was a linear function of both time and protein concentration at rates of less than 0.05 μmole of DPNH (or TPNH) formed per minute. A unit of activity was defined as that amount of transaminase allowing the formation of 1 μmole of reduced pyridine nucleotide per minute in the standard assay system. Specific activity was expressed in terms of units per mg. of protein.

Growth—The organism was maintained on agar (1.5 per cent) slants containing inorganic salts (11) supplemented with 0.4 per cent of previously neutralized pyrrolidine. For large scale growth the surface of an agar slant culture was washed and the washings were used to inoculate a 6-l. flask containing 1.5 l. of the same medium which did not contain agar. After 24 hours of incubation at approximately 28° with vigorous mechanical shaking the culture was used to inoculate 90 l. of the inorganic salts medium containing 1 per cent of yeast extract, 0.8 per cent of neutralized pyrrolidine and 0.001 per cent of Dow-Corning AF antifoam emulsion. The medium was distributed among six 5-gallon carboys equipped for vigorous aeration. Growth was allowed to take place at approximately 25° for 16 hours at which time the cells were harvested with a Sharples centrifuge, washed twice with 5 volumes of 0.9 per cent sodium chloride, and frozen. The yield of cells varied between 100 to 275 gm., wet weight, per 90 l. As previously noted (8), the enzyme is present in cells produced with pyrrolidine or γ-aminobutyrate in the medium and absent when these compounds are omitted.

Purification—After the cells were thawed, they were washed with 10 volumes of 0.1 M phosphate buffer[3] (pH 7.35) containing 0.01 per cent mercaptoethanol. After centrifugation, the supernatant fluid was discarded. This freezing, thawing, and washing procedure removed large amounts of nucleic acid and allowed a better subsequent fractionation with protamine and

[1] The enzymatic conversion of Δ^1-pyrroline to γ-aminobutyraldehyde and the oxidation of the latter by a DPN-linked enzyme will be the subject of a future report.

[2] It is a pleasure to acknowledge the generous amounts of purified yeast-DPN aldehyde dehydrogenase obtained from Dr. S. Black. The preparation of a fraction with succinic semialdehyde dehydrogenase activity is detailed under "Materials and Methods."

[3] Unless otherwise noted, references to phosphate buffer refer to solutions of potassium phosphate at pH 7.35 containing 0.01 per cent 2-mercaptoethanol.

salt. The residue was dispersed in an equal volume of 0.1 M phosphate buffer and treated in a 10 kc. Raytheon sonic oscillator for 5 minutes. The entire suspension was diluted with 2 volumes of the same buffer and centrifuged for 20 minutes at approximately 14,000 × *g*. The residue was again treated in the same manner including sonic oscillation for 5 minutes and the resulting residue was discarded. The two supernatant fluids were combined and represent the crude extract (Fraction 1).

To the extract was added a 1 per cent solution of protamine sulfate so that 12.5 mg. were used per gm. of cells. After removal of the precipitate 35 gm. of ammonium sulfate were added per 100 ml. of the supernatant fluid and the precipitate was discarded. The material salting out upon the further addition of 21 gm. of ammonium sulfate was removed by centrifugation and dissolved in 0.1 M potassium phosphate to yield Fraction 2.

To Fraction 2 was added sufficient 2-mercaptoethanol to provide a concentration of 0.1 per cent. This solution was poured into 10 volumes of previously cooled (−15°) acetone and the resulting suspension filtered with suction. The precipitate was thoroughly suspended in a small volume of 0.1 M phosphate buffer and the insoluble material removed by centrifugation and discarded. The supernatant fluid was brought to a mercaptoethanol concentration of 0.05 per cent and dialyzed overnight against 100 volumes of 0.05 M phosphate buffer. After dialysis insoluble material was removed by centrifugation (Fraction 3).

The dialyzed solution was brought to a mercaptoethanol concentration of 0.1 per cent and an equal volume of precooled acetone (−15°) added. The precipitate which formed was saved for the purification of succinic semialdehyde dehydrogenase (9). To the supernatant fluid was added sufficient acetone to yield a final concentration of 75 per cent (volume for volume). The precipitate thus formed was dissolved in a small volume of 0.01 M potassium phosphate buffer (Fraction 4).

Fraction 4 was treated repeatedly with small amounts of calcium phosphate gel. A mg. of gel was added per mg. of protein in Fraction 4. The gel was removed by centrifugation and the process repeated until 90 per cent of the enzyme activity had been adsorbed. The first several gel treatments adsorbed little enzyme and these fractions were discarded. The gel fractions containing the bulk of enzyme were combined and eluted with 0.1 M potassium phosphate buffer. The eluate represents Fraction 5.

To Fraction 5 were added 42 gm. of ammonium sulfate per 100 ml. of eluate and the resultant precipitate was removed by centrifugation and discarded. The precipitate formed by the addition of another 10.5 gm. of ammonium sulfate was dissolved in 0.05 M phosphate buffer (Fraction 6).

A typical protocol (Table I) summarizes the results of one such preparation. When stored at −20° preparations of Fractions 2 and 6 appear to be stable for several months. At pH values above 7.5 and below 6.5, however, the enzyme is rapidly inactivated.

RESULTS

Specificity and Products—The transaminase is active only with the reactants described by Reaction 1. Negative results were obtained when the following substitutions were made: β-alanine, δ-aminovaleric acid, ε-aminocaproic acid, ω-aminocaprylic acid, ornithine, or lysine for γ-aminobutyric acid; pyruvic acid, oxaloacetic acid, ketomalonic acid or α-ketoisovaleric acid for α-ketoglutaric acid; aspartic acid, L-2,4-diaminoglutaric acid, alanine, or D-glutamic acid for L-glutamic acid; glyoxylic acid, glycolaldehyde, or malonic semialdehyde for succinic semialdehyde.

TABLE I

Summary of typical purification

163 gm. of cells (wet weight) were used.

Fraction treatment	Volume	Total activity	Total protein	Specific activity
	ml.	*units*	*mg.*	*units/mg.*
1. Extract	1,040	628	10,400	0.06
2. Protamine, ammonium sulfate	52	415	1,150	0.36
3. Acetone	50	308	720	0.43
4. Dialysis, acetone	17	126	152	0.83
5. Gel eluate	53	69	39	1.75
6. Ammonium sulfate	3.2	35	10.5	3.40

The products of the reaction at equilibrium were identified by paper chromatography with a butanol-acetic acid-water (4:1:1) solvent. Identical products were obtained starting with γ-aminobutyrate and α-ketoglutarate or with succinic semialdehyde and L-glutamate. The R_F values of the four substrates were as follows: γ-aminobutyrate, 0.41; α-ketoglutarate, 0.25; succinic semialdehyde, 0.82; glutamate, 0.19. The amino acid areas were developed with ninhydrin while silver nitrate was used to demonstrate the keto acid and aldehyde.

Evidence that the reaction under discussion was a transamination was obtained by an experiment in which DL-glutamate-2-C^{14} (0.25 μmoles containing 95,000 c.p.m.) was incubated with an excess of succinic semialdehyde in the presence of the enzyme. Chromatography of the reaction products resulted in the finding of radioactivity in the glutamate (presumably largely D-glutamate) and α-ketoglutarate areas of the chromatogram. The γ-aminobutyrate which was formed was not radioactive.

Equilibrium Constant—The results of experiments to determine the equilibrium constant are shown in Table II. When the

TABLE II

Equilibrium constant at pH 8.1 and 38°

The reaction mixture contained 1 unit of enzyme and 100 μmoles of pyrophosphate buffer at pH 8.1 per ml. as well as the substrates listed in the table. After 2 hours at 38° aliquots of 0.1 ml. were added to 0.1 ml. of 1 N HCl to stop the reaction. Samples taken at subsequent time intervals yielded values identical to those from the 2-hour samples. To the acid samples were added 0.1 ml. of 1 N NaOH, 100 μmoles of potassium phosphate at pH 7.35, 0.5 μmole of DPN and the yeast dehydrogenase active on succinic semialdehyde. The total change in optical density resulting upon the addition of the dehydrogenase was used as a measure of succinic semialdehyde concentration. The abbreviations γAB, αKG, SSA, and G refer to γ-aminobutyrate, α-ketoglutarate, succinic semialdehyde, and glutamate, respectively.

Initial concentration (mM)				Equilibrium concentration (mM) SSA	$K_{eq.}$
γAB	αKG	SSA	G		
2.00	1.00			.33	0.10
1.00	1.00			.24	0.10
1.00	2.00			.33	0.10
		1.00	2.00	.11	0.15
		1.00	1.00	.28	0.15
		2.00	1.00	1.16	0.26

reaction was started with succinic semialdehyde and glutamate the amount of succinic semialdehyde at equilibrium was always somewhat higher than when starting with γ-aminobutyrate and α-ketoglutarate. The reason for this finding is not clear. However, since the semialdehyde was not purified to the same degree as the other substrates the equilibrium values starting with γ-aminobutyrate are considered to be the most reliable and in fact agree best with one another. From the latter value the ΔF of the reaction was calculated to be 1.4 kcal. per mole.

The equilibrium constant (Table III) was independent of temperature between 20 and 38° and of pH between 7.4 and 8.8.

Substrate Concentration—The relation of initial velocity to substrate concentration is shown in Fig. 1. A competitive inhibition due to α-ketoglutarate was found which was more evi-

TABLE III

Effect of temperature and pH upon equilibrium

A μmole each of γ-aminobutyrate and α-ketoglutarate were added to initiate the reaction. Other conditions were as in Table II.

Temperature	pH	Succinic semialdehyde concentration at equilibrium
°C		mM
20	8.1	0.24
30	8.1	0.23
38	8.1	0.23
25	7.4	0.24
25	7.8	0.24
25	8.4	0.24
25	8.8	0.25

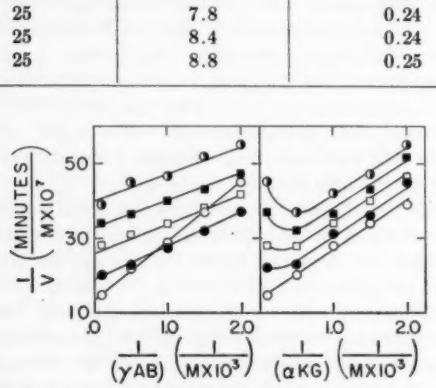


FIG. 1. Dependence of initial velocity, v, on substrate concentration at pH 8.8 and 25°. The reaction mixture contained the transaminase and the following other components in 1.0 ml: 100 μmoles of potassium pyrophosphate, 0.5 μmole of DPN, an excess of yeast aldehyde dehydrogenase, and the concentrations of α-ketoglutarate (αKG) and γ-aminobutyrate (γAB) indicated.

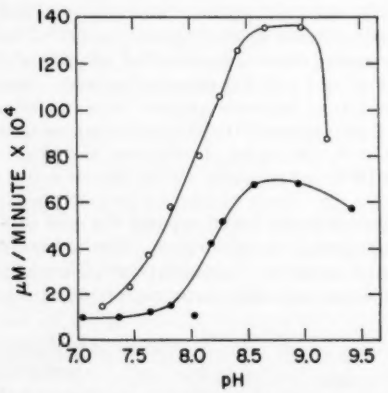


FIG. 2. Dependence of reaction velocity on pH at 25° and 1.0 mM substrate concentration. O——O, α-ketoglutarate and γ-aminobutyrate as the substrates; ●——●, glutamate and succinic semialdehyde as the substrates. In the former case the velocity of the reaction was determined as in Fig. 1 and represents initial rates. In the latter case, rates were determined by the method used in Table II. Pyrophosphate buffers at a concentration of 0.1 M were employed.

TABLE IV

Effect of pH at 25° on constants of Equation 3

Conditions are those described for Fig. 1, with pyrophosphate buffer used in each case. K_a' was calculated from K_a assuming that only the unionized amino form of γ-aminobutyric acid reacts with the enzyme. The units of the constants are: $1/V_{max}$, minutes per mole; K_b, K_a, and K_a', minutes per liter; $1/K_I$, liters per mole.

pH	$1/V_{max}$	K_b	K_a	$1/K_I$	K_a'
8.83	12 × 10⁷	14 × 10⁴	6.4 × 10⁴	150	1200
8.42	11 × 10⁷	12 × 10⁴	12 × 10⁴	200	920
8.08	12 × 10⁷	14 × 10⁴	25 × 10⁴	210	850
7.45	12 × 10⁷	14 × 10⁴	120 × 10⁴	240	960

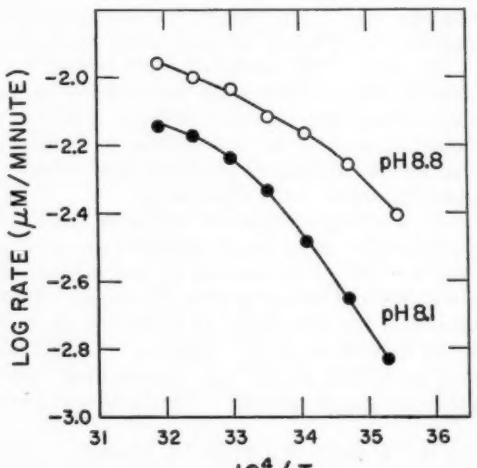


FIG. 3. Dependence of initial velocity on temperature at 1.0 mM substrate concentration. Rates were determined as in Fig. 1.

TABLE V

Effects of temperature at pH 8.83 on constants of Equation 3

The conditions are those used in Fig. 1. K_a' was calculated as in Table IV. The units of the constants are defined in Table IV.

Temperature	$1/V_{max}$	K_b	K_a	$1/K_I$	K_a'
°C					
40	5 × 10⁷	16 × 10⁴	2.9 × 10⁴	90	1500
30	9 × 10⁷	15 × 10⁴	4.5 × 10⁴	120	1300
25	12 × 10⁷	14 × 10⁴	6.4 × 10⁴	150	1300
20	17 × 10⁷	13 × 10⁴	8.0 × 10⁴	250	1200
10	35 × 10⁷	11 × 10⁴	15.0 × 10⁴	380	1100

dent at lower temperatures or at the lower pH values. The results appear to satisfy the equation.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_k}{[\alpha KG]} + \frac{K_a}{[\gamma AB]}\left(1 + \frac{[\alpha KG]}{K_I}\right) \quad (3)$$

where v is the initial velocity, V_{max} the maximum velocity, αKG and γAB the concentration of α-ketoglutarate and γ-aminobutyrate, respectively, K_k and K_a constants whose meaning will be discussed, and K_I the inhibition constant for α-ketoglutarate.

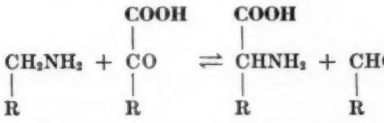
pH—The dependence of the initial reaction rate on pH under standard assay conditions, *i.e.* 1 mM substrates and 25°, is depicted in Fig. 2. At four different pH values the kinetic constants of Equation 3 were determined with the results shown in Table IV.

Temperature—The temperature dependence of initial rates of the reaction under standard conditions at two pH values is shown in Fig. 3. The kinetic constants of Equation 3 were determined at five different temperatures and the results are summarized in Table V.

Cofactors—No direct evidence for the participation of any cofactors could be demonstrated. The addition of pyridoxal phosphate to crude or purified and dialyzed enzyme preparations or preparations subjected to extremes of pH was without effect. However, in common with other transaminases and pyridoxal phosphate activated enzymes, the enzyme was inhibited by carbonyl reagents. Potassium cyanide and hydroxylamine caused 50 per cent inhibition at concentrations of 1×10^{-5} M and 3×10^{-6} M, respectively.

DISCUSSION

Reaction 1 has been rephrased in Reaction 4 where R represents —CH_2CH_2COOH.



(4)

Written in this manner it becomes conceivable that the reaction could be one of transcarboxylation rather than transamination. That transcarboxylation is not the case has been demonstrated by incubation of the enzyme with glutamate-2-C^{14} and succinic semialdehyde. The absence of radioactivity in the γ-aminobutyrate thus formed is taken as evidence that the enzyme under discussion is a transaminase.

Equation 3 has been developed by Alberty (12) for the kinetic analysis of reactions in which two substrates are involved and where the formation and decomposition of binary complexes of the enzyme occur. In the case of certain dehydrogenases where ternary complexes appear to participate a different relationship has been found (13, 14, 9). A mechanism for transamination which involves only binary complexes strongly suggests that the enzyme itself transaminates with each substrate in turn. If the γ-aminobutyric-glutamic transaminase, in analogy with other transaminases (15), has pyridoxal phosphate tightly bound as a prosthetic group, the reaction is most simply summarized as shown in Reactions 5 to 9:

$$RCH_2NH_2 + ECHO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E' \quad (5)$$

$$E' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RCHO + ECH_2NH_2 \quad (6)$$

$$RCOCOOH + ECH_2NH_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'' \quad (7)$$

$$E'' \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} RCH_2NH_2COOH + ECHO \quad (8)$$

$$ECHO + RCOCOOH \underset{k_{-I}}{\overset{k_I}{\rightleftharpoons}} E''' \quad (9)$$

in which E is the enzyme, and E′, E″, and E‴ substrate-enzyme complexes of unspecified structure. The enzyme is pictured as bearing pyridoxal phosphate as a tightly bound prosthetic group. It is proposed that the prosthetic group transaminates with each substrate in turn thus involving two forms of the holo-enzyme; an aldehyde (ECHO) and an amino (ECH_2NH_2) form. Based on these assumptions the constants in Equation 3 become: $1/V_{max} = 1/k_2 + 1/k_4$; $K_k = (k_4 + k_{-3})/k_3k_4$; $K_a = (k_2 + k_{-1})/k_1k_2$. If further intermediate steps are assumed the interpretation of these constants would, of course, be different.

Between pH 7.4 and 8.8 there is little effect of pH on the maximal velocity, on K_k, or on the inhibition constant K_I (Table IV). If it is assumed that only the unionized amino group of γ-aminobutyrate reacts then a corrected value, K_a', is also almost constant. The values of K_a' have been calculated from K_a assuming that only the unionized form of γ-aminobutyric acid is active and that the pK_b of this compound is 10.55 at 25° (16).

Only K_k is relatively independent of temperature (Table V). The maximal velocity of the reaction varies with temperature in such a manner as to indicate an apparent activation energy of 11.5 kcal. per mole. K_I shows a similar dependence with an apparent heat of dissociation of 10 kc. per mole. The variation of K_a with temperature can be almost entirely explained by the high heat of dissociation (approximately 10 kcal. per mole (16)) of the amino group of γ-aminobutyrate. Again, assuming that only the unionized amino group is active, K_a' has a slight dependence on temperature similar to that of K_k.

MATERIALS AND METHODS

The diethyl ester of 2-formylsuccinic acid was obtained as a gift from Dr. R. W. Albers and was used for the preparation of succinic semialdehyde by a method described by Bessman *et al.* (3). The diethyl acetal of malonic semialdehyde ethyl ester was a gift from Dr. M. J. Coon and Dr. W. G. Robinson and was used for the preparation[4] of malonic semialdehyde. L-Glutamic acid was a gift from Dr. J. P. Greenstein and L-2,4-diaminoglutaric acid from Dr. H. N. Christensen. γ-Aminobutyric acid was recrystallized twice before use. All other compounds employed are commercially available. Calcium phosphate gel (17) contained 15 mg. (dry weight) per ml.

Protein was determined by the method of Lowry *et al.* (18) with the use of crystalline bovine serum albumin as a standard. Radioactivity was measured with a Forro chromatogram scanner and a recording rate meter. Enzyme assays were performed with a Beckman model DU spectrophotometer equipped for temperature regulation.

[4] Personal communication from Dr. M. J. Coon.

For preparation of the yeast dehydrogenase acting on succinic semialdehyde, purified DPN-aldehyde dehydrogenase brought to the stage of Fraction 3 (10) by Dr. S. Black was further purified with ammonium sulfate. Ammonium sulfate, 28 gm., was added per 100 ml. of the protein solution and the resultant precipitate was discarded. The precipitate formed after an additional 21 gm. were added, was dissolved in 0.05 M phosphate buffer at pH 7.0 containing 0.1 per cent mercaptoethanol. One such preparation, active in the oxidation of succinic semialdehyde, was inactive with acetaldehyde as substrate.

SUMMARY

A soluble γ-aminobutyric-glutamic transaminase has been purified from extracts of a strain of *Pseudomonas fluorescens* which had been shown to catalyze a reversible transamination with γ-aminobutyric acid and α-ketoglutaric acid as substrates yielding succinic semialdehyde and glutamic acid.

A kinetic analysis was consistent with the hypothesis that the reaction proceeds by the formation and decomposition of a series of binary complexes of enzyme and substrate. The data suggest that the unionized form of γ-aminobutyric acid is the reactive species.

REFERENCES

1. Awafara, J., Landua, A. J., Fuerst, R., and Seale, B., *J. Biol. Chem.*, **187**, 35 (1950).
2. Roberts, E., and Frankel, S., *J. Biol. Chem.*, **187**, 55 (1950).
3. Bessman, S. P., Rossen, J., and Layne, E. C., *J. Biol. Chem.*, **201**, 385 (1953).
4. Roberts, E., and Bregoff, H. M., *J. Biol. Chem.*, **201**, 393 (1953).
5. Roberts, E., Ayengar, P., and Posner, I., *J. Biol. Chem.*, **203**, 195 (1953).
6. Kinney, R. W., and Werkman, C. H., *Bacteriol. Proc.*, 118 (1958).
7. Noe, F. F., and Nickerson, W. J., *J. Bacteriol.*, **75**, 674 (1958).
8. Scott, E. M., and Jakoby, W. B., *Science*, **128**, 361 (1958).
9. Jakoby, W. B., and Scott, E. M., **234**, 937 (1959).
10. Black, S., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. I*, Academic Press, Inc., New York, 1955, p. 508.
11. Jakoby, W. B., *J. Biol. Chem.*, **232**, 75 (1958).
12. Alberty, R. A., *J. Am. Chem. Soc.*, **75**, 1932 (1953).
13. Theorell, H., *Discussions Faraday Soc.*, **20**, 224 (1955).
14. Hakala, M. T., Glaid, A. J., and Schwert, G. W., *J. Biol. Chem.*, **221**, 191 (1956).
15. Meister, A., *Advances in Enzymol.*, **16**, 185 (1955).
16. Edsall, J. T., and Wyman, J., *Biophysical chemistry, Vol. I*, Academic Press, Inc., New York, 1958, p. 453.
17. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London B*, **124**, 397 (1938).
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).

Aldehyde Oxidation

III. SUCCINIC SEMIALDEHYDE DEHYDROGENASE

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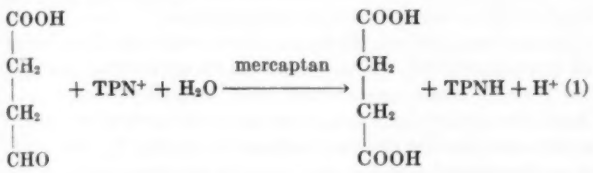
(Received for publication, October 14, 1958)

A particulate preparation from brain (1) and a soluble, purified enzyme from *Pseudomonas fluorescens* (2) have been shown to catalyze the transamination of γ-aminobutyric acid with α-ketoglutaric acid resulting in the formation of succinic semialdehyde and glutamic acid. The further utilization of succinic semialdehyde was indicated by the finding that this compound may be oxidized through the mediation of pyridine nucleotide-linked dehydrogenases isolated simultaneously from the pseudomonad (3) and mammalian brain (4). The present report is concerned with the purification and the properties of the enzyme, succinic semialdehyde dehydrogenase, from *P. fluorescens*. The dehydrogenase catalyzes Reaction 1.



In the presence of the enzyme, mercaptan, and TPN (or, at a slower rate, DPN), succinic semialdehyde is quantitatively converted to succinic acid.

The dehydrogenase, in conjunction with γ-aminobutyric-glutamic transaminase, has been found to be useful for the quantitative determination of γ-aminobutyrate and α-ketoglutarate. A method for the determination of these compounds is presented.

PREPARATION OF ENZYME

The organism (2, 3) was allowed to grow on a medium containing pyrrolidine, and the enzyme was extracted as described for γ-aminobutyric-glutamic transaminase by Scott and Jakoby (2).

Assay—Enzyme activity was followed spectrophotometrically at 340 mμ by utilizing the absorption band of reduced pyridine nucleotide. To an appropriate amount of enzyme in a total volume of 1.0 ml. were added the following (in μmoles): tris(hydroxymethyl)aminomethane chloride at pH 7.9, 50; 2-mercaptoethanol, 3; TPN, 1. The reaction was initiated after a preincubation period of 40 minutes by the addition of 0.5 μmoles of succinic semialdehyde and the optical density change was followed with a Beckman DU spectrophotometer at 30-second intervals for 3 minutes. Alternatively a Cary recording spectrophotometer was employed. The rate of TPNH formation under these conditions was linear, both with respect to time and enzyme concentration, when optical density changes of less than 0.3 per minute were measured. A unit of dehydrogenase activity is defined as that amount of enzyme catalyzing the formation of 1.0 μmole of TPNH per minute in the standard incubation mixture described above. Specific activity is defined in terms of units per mg. of protein.

Purification—The early steps in the purification, including protamine treatment, ammonium sulfate fractionation, and treatment with a large volume of acetone were those described for γ-aminobutyric-glutamic transaminase (2). The dialyzed solution (41 ml., Fraction 4, Table I, below) was brought to a mercaptoethanol concentration of 0.1 per cent and an equal volume of cooled (−15°) acetone was added. The precipitated material was suspended in 21 ml. of 0.05 M tris(hydroxymethyl)-aminomethane buffer at pH 7.2 containing 0.1 per cent mercaptoethanol. Two mg. of calcium phosphate gel were added per ml. of the resultant suspension and the residue after centrifugation was discarded. The clear supernatant fluid (22 ml.) represents Fraction 5. Fraction 5 was acidified to pH 5.2 with 1 N acetic acid and treated with 2 mg. of calcium phosphate gel per mg. of protein. Before use the gel has been centrifuged and the suspending fluid discarded. Thus the calcium phosphate was suspended directly in Fraction 5. After centrifugation of this suspension the gel was eluted successively with 0.1 M tris(hydroxymethyl)aminomethane at pH 7.2 and with 0.07 M phosphate at pH 7.0, each of which contained 0.1 per cent mercaptoethanol. The volume of eluent used was twice the volume of Fraction 5. The phosphate eluate contained the enzyme activity (Fraction 6).

Per 10 ml. of Fraction 6 3.5 gm. of ammonium sulfate were added and the small precipitate which formed was removed by centrifugation. To the supernatant fluid 1.73 gm. of ammonium sulfate were added and the resulting precipitate was dissolved in 0.05 M potassium phosphate at pH 7.0 containing 0.1 per cent mercaptoethanol (Fraction 7).

The summary of a typical preparation is presented in Table I. Attempts at further purification by chromatography on *N,N*-diethylaminoethylcellulose (5) produced a 2-fold increase in specific activity but resulted in a 95 per cent loss of total enzyme activity.

PROPERTIES OF ENZYME

Succinic semialdehyde dehydrogenase was equally active in an air or in a helium atmosphere. The enzyme displayed maximal activity at pH 8.5 (Fig. 1) and was most stable at pH 6.5 to 7.1. Preparations stored at pH 7.0 in 5 mM mercaptoethanol-0.05 M

TABLE I

Purification of succinic semialdehyde dehydrogenase from Pseudomonas

Ninety-five gm. of cells (wet weight) were used.

Fraction	Procedure	Volume	Total Activity	Total Protein	Specific Activity
		ml.	*units*	*mg.*	*units/mg.*
1	Extract	550	869	12,100	0.07
2	Protamine	630	1,065	8,700	0.12
3	Ammonium sulfate	41	825	1,500	0.55
4	Acetone, dialysis	40	721	840	0.86
5	Acetone	22	710	506	1.40
6	Gel eluate	41	371	123	3.05
7	Ammonium sulfate	5	338	92	3.61

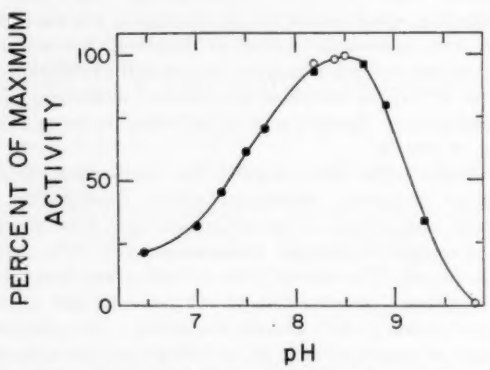


FIG. 1. Dependence of dehydrogenase activity on pH. All buffers were 0.1 M; (●) potassium phosphate, (○) tris(hydroxymethyl)aminomethane chloride, (■) glycine.

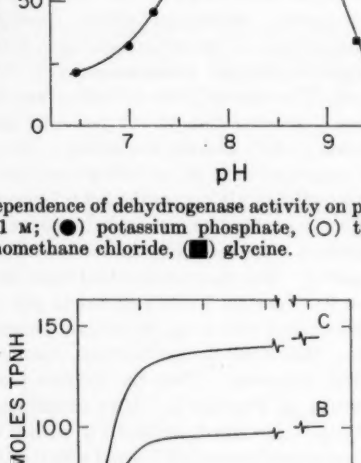


FIG. 2. Tracing from a Cary spectrophotometer illustrating the stoichiometry of the dehydrogenase reaction. The curves represent the formation of TPNH as a function of time following the addition of 50 (*A*), 100 (*B*) and 150 (*C*) mμmoles of succinic semialdehyde, respectively.

phosphate buffer at 2° lost 10 per cent of their catalytic activity per week.

Specificity and Stoichiometry—These preparations were specific for the oxidation of succinic semialdehyde. Thus, glyoxylate and malonic semialdehyde[1] in comparable concentrations did not lead to the formation of reduced pyridine nucleotide in this system. A wide variety of aliphatic, aromatic, and heterocyclic aldehydes which had previously been shown to be oxidized by an aldehyde dehydrogenase from a different strain of *P. fluorescens* (7), was not utilized by succinic semialdehyde dehydrogenase. Since it has been reported (8) that succinic semialdehyde is oxidized by glyceraldehyde 3-phosphate dehydrogenase, glyceraldehyde 3-phosphate was tested as a substrate for succinic semialdehyde dehydrogenase. No reduction of pyridine nucleotide occurred and it was concluded that the *Pseudomonas* enzyme is not a glyceraldehyde 3-phosphate dehydrogenase.

Succinic semialdehyde was oxidized completely (Fig. 2) and reversal of the reaction could not be demonstrated. Succinic acid was identified as the product by chromatographing ether extracts of an incubation mixture in which 4 μmoles of succinic semialdehyde had been utilized. The isoamyl alcohol-4 M formic acid solvent system described by Flavin and Ochoa (9) was used. Purified enzyme preparations treated with Dowex 1 (Cl) resin and with activated charcoal (10) were not stimulated by coenzyme A or ATP nor was a hydroxamic acid-reacting material formed either in the presence of coenzyme A and ATP or with coenzyme A and inorganic phosphate.

Both TPN and DPN served as substrates although the former was 8.2 times more active under standard assay conditions, *i.e.* at a nucleotide concentration of 1 mM. This ratio was constant throughout the last stages of purification. In the first four steps of purification, accurate assays could not be obtained with the use of DPN because of the presence of a system reducing DPN in the absence of succinic semialdehyde.

After storage for several days or fractionation in the absence of mercaptoethanol, enzyme preparations were found to have low activity unless preincubated with sulfhydryl compounds. Activation occurred at room temperature (23°) over a 40-minute period as shown for one such preparation in Fig. 3. Although mercaptoethanol, cysteine, and glutathione were equally effective at a concentration of 5 mM, mercaptoethanol was used routinely as a convenient source of exogenous mercaptan.

Kinetics—The results of a kinetic analysis of Reaction 1 are consistent with the involvement of a ternary complex involving the enzyme, succinic semialdehyde and TPN. The velocity of the reaction may be expressed (11) in terms of Equation 1[2] where K_a, K_b and K_c are constants and V_M is the maximal velocity at a given enzyme concentration. For the conditions employed in Fig. 4, *i.e.*, pH 7.9

$$\frac{V_M}{v} = 1 + \frac{K_a}{[\text{TPN}]} + \frac{K_b}{[\text{succinic semialdehyde}]} + \frac{K_c}{[\text{TPN}]\,[\text{succinic semialdehyde}]} \quad (1)$$

and 25°, the rate of the reaction is represented by Equation 2.

$$\frac{V_M}{v} = 1 + \frac{2.8 \times 10^{-6}}{[\text{TPN}]} + \frac{5.6 \times 10^{-6}}{[\text{succinic semialdehyde}]} + \frac{3.4 \times 10^{-9}}{[\text{TPN}]\,[\text{succinic semialdehyde}]} \quad (2)$$

It will be evident from the data presented in Fig. 4 that the experimental points are in good agreement with the curves cal-

[1] Malonic semialdehyde was used as such or generated *in situ* with acetylene monocarboxylic acid hydrase and its substrate (6).

[2] The units of the constants are: K_a and K_b, moles per l.; K_c, (moles per l.)^2.

culated from Equation 2. The simplest mechanism compatible with the results is in agreement with the mechanisms proposed for alcohol dehydrogenase (12) and lactic dehydrogenase (13) and is summarized by Reactions 2 to 6. As suggested by Alberty (11) the assumption is made here

$$E + (TPN) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(TPN) \quad (2)$$

$$E(TPN) + (\text{succinic semialdehyde}) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (\text{succinic semialdehyde})E(TPN) \quad (3)$$

$$E + (\text{succinic semialdehyde}) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (\text{succinic semialdehyde})\,E \quad (4)$$

$$(\text{succinic semialdehyde})E + (TPN) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} (\text{succinic semialdehyde})E(TPN) \quad (5)$$

$$(\text{succinic semialdehyde})E(TPN) \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E\,(\text{products}) \quad (6)$$

that Reaction 6 is rate-limiting. Based on this mechanism the constants of Equation 1 become $K_a = k_3(k_2 + k_4)/(k_3 - k_1)$; $K_b = k_1(k_2 + k_4)/(k_3 - k_1)$; $K_c = k_1k_3(k_2 + k_4)/(k_3 - k_1)$.

Inhibitions—Two semialdehydes, glyoxylate and malonic semialdehyde, were found to be competitive inhibitors of the enzyme. The K_i for these compounds at a TPN concentration of 1×10^{-3} M was calculated as 3×10^{-4} M for glyoxylic acid and 1×10^{-4} M for malonic semialdehyde. The following compounds at the concentrations noted did not inhibit: glycolaldehyde, 2×10^{-3} M; succinic acid, 1×10^{-2} M; ethylenediaminetetracetic acid, 1×10^{-2} M. In common with other aldehyde dehydrogenases (14) the succinic semialdehyde dehydrogenase was inhibited by relatively low concentrations of arsenite; 50 per cent inhibition was obtained at a potassium arsenite concentration of 4×10^{-4} M.

α-KETOGLUTARATE AND γ-AMINOBUTYRATE ASSAY

By coupling succinic semialdehyde dehydrogenase (Reaction 1) with γ-aminobutyrate-glutamate transaminase (2) (Reaction 7) a sensitive and specific assay for α-ketoglutarate and γ-aminobutyrate has been developed. The net result of coupling the dehydrogenase and the transaminase is summarized by Reaction 8.

$$\gamma\text{-aminobutyrate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{succinic semialdehyde} + \text{glutamate} \quad (7)$$

$$\gamma\text{-aminobutyrate} + \alpha\text{-ketoglutarate} + TPN^+ + H_2O \rightleftharpoons \text{succinate} + \text{glutamate} + TPNH + H^+ \quad (8)$$

The assay system for either of these metabolites consisted of the following (in μmoles) in a total volume of 1.0 ml.: tris(hydroxymethyl)aminomethane at pH 7.9, 50; 2-mercaptoethanol, 5; TPN, 1; sufficient succinic semialdehyde dehydrogenase and γ-aminobutyric-glutamic transaminase to result in a rate of optical density change of approximately 0.1 per minute. For assay of α-ketoglutarate the reaction mixture was supplemented with 2 μmoles of γ-aminobutyrate and for the determination of γ-aminobutyrate, 2 μmoles of α-ketoglutarate were added. The amount of either of these metabolites present in a sample was deduced from the increase in optical density at 340 mμ ($\epsilon = 6.21 \times 10^3$ (15)) due to the formation of TPNH. Extensive purification of the enzymes was not necessary since it had been found that Fraction 2, the first ammonium sulfate fraction, contained both the dehydrogenase and the transaminase and was well suited for these assays. Glutamic acid does not interfere; glutamic dehydrogenase is absent. This system is most useful for final concentrations of samples containing between 1.5×10^{-5} M to 1.5×10^{-4} M. Within this range repeated determinations result in variations of less than 3 per cent.

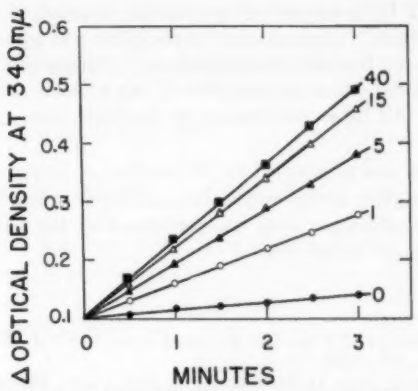


FIG. 3. Effect of preincubation with mercaptoethanol on enzyme activity. The figures to the right of the curves indicate the time of preincubation. The reaction was initiated by the addition of succinic semialdehyde.

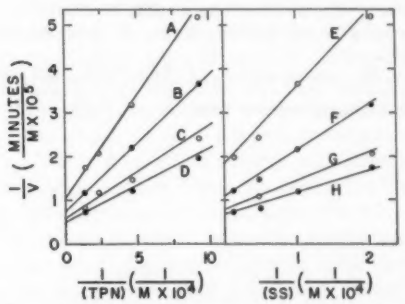


FIG. 4. Dependence of initial velocity, V, on substrate concentration at pH 7.9. The reaction mixture contained the enzyme (specific activity 3.8), 50 μmoles of tris(hydroxymethyl)aminomethane chloride, and 5 μmoles of mercaptoethanol. The succinic semialdehyde concentrations were 49, 98, 245, and 745 μM for *Curves A*, *B*, *C* and *D*, respectively. The TPN concentrations were 10.8, 21.6, 43.2, and 72.0 μM for *Curves E*, *F*, *G* and *H*, respectively. The curves have been calculated from Equation 2.

MATERIALS AND METHODS

The diethyl ester of 2-formylsuccinic acid, obtained from Dr. R. W. Albers, was hydrolyzed in dilute solution (0.1 M) by heating in a boiling water bath for 4 hours with 0.2 N HCl. The resulting solution, containing succinic semialdehyde, was adjusted to pH 6.0 with KOH. The diethyl acetal of malonic semialdehyde ethyl ester was a gift from Dr. M. J. Coon and

Dr. W. G. Robinson and was used for the preparation[3] of malonic semialdehyde. Glyceraldehyde 3-phosphate was generated with aldolase and fructose 1,6-diphosphate. Calcium phosphate gel was prepared (16) so as to contain 21 mg. of solids (dry weight) per ml. All other compounds are available from commercial sources.

Protein was determined by the method of Lowry *et al.* (17) with crystalline bovine serum albumin used as a standard. Assay for hydroxamic acids was performed by the technique of Lipmann and Tuttle (18).

SUMMARY

Succinic semialdehyde dehydrogenase has been purified from extracts of *Pseudomonas fluorescens*. The enzyme is specific for succinic semialdehyde oxidation and forms succinic acid as the product. Triphosphopyridine nucleotide or diphosphopyridine nucleotide as well as a mercaptan are required for the reaction.

Simple and specific assays for γ-aminobutyrate and for α-ketoglutarate in the range of 1.5×10^{-5} M to 1.5×10^{-4} M have been developed which use γ-aminobutyric-glutamic transaminase coupled with succinic semialdehyde dehydrogenase.

REFERENCES

1. Bessman, S. P., Rossen, J., and Layne, E. C., *J. Biol. Chem.*, **201**, 385 (1953).
2. Scott, E., and Jakoby, W. B., *J. Biol. Chem.*, **234**, 932 (1959).
3. Scott, E., and Jakoby, W. B., *Science*, **128**, 361 (1958).
4. Albers, R. W., and Salvadore, R. A., *Science*, **128**, 359 (1958).
5. Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 756 (1956).
6. Yamada, E. W., and Jakoby, W. B., *J. Biol. Chem.*, **234**, 941 (1959).
7. Jakoby, W. B., *J. Biol. Chem.*, **232**, 75 (1958).
8. Harting, J., and Velick, S. F., *J. Biol. Chem.*, **207**, 857 (1954).
9. Flavin, M., and Ochoa, S., *J. Biol. Chem.*, **229**, 965 (1957).
10. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191**, 365 (1951).
11. Alberty, R. A., *J. Am. Chem. Soc.*, **75**, 1928 (1953).
12. Theorell, H., *Discussions Faraday Soc.*, **20**, 224 (1955).
13. Hakala, M. T., Glaid, A. J., and Schwert, G. W., *J. Biol. Chem.*, **221**, 191 (1956).
14. Jakoby, W. B., *J. Biol. Chem.*, **232**, 89 (1958).
15. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175**, 385 (1948).
16. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London B*, **124**, 397 (1938).
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
18. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).

[3] Personal communication from Dr. M. J. Coon.

Enzymatic Utilization of Acetylenic Compounds

II. ACETYLENEMONOCARBOXYLIC ACID HYDRASE

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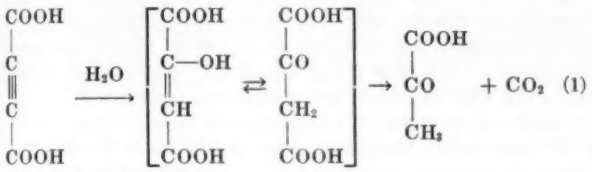
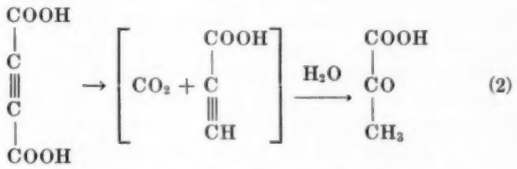
(Received for publication, October 14, 1958)

Previous work on the utilization of acetylenic compounds led to the finding of an enzyme that catalyzes the hydration and decarboxylation of acetylenedicarboxylic acid with the formation of pyruvic acid (1, 2). The reaction sequence may be considered to occur by Reactions 1 or 2, although the proposed intermediates, in each case, were inactive in the free state.

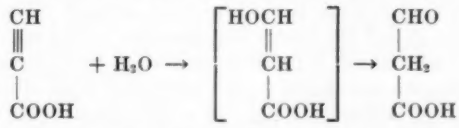


(1)



(2)

It was considered that the separation and study of an enzyme utilizing acetylenemonocarboxylic acid (propiolic acid) might yield information useful in evaluating the mechanisms described by Reactions 1 and 2. Accordingly a bacterium, with the ability to grow on acetylenemonocarboxylic acid as the sole carbon source, was isolated. An enzyme was purified from extracts of this organism that was able to catalyze the formation of malonic semialdehyde from acetylenemonocarboxylic acid. This conversion is considered to proceed according to Reaction 3.



(3)

The data on the purification and the properties of the enzyme catalyzing Reaction 3 are presented here, and the pertinence of these findings to the acetylenedicarboxylic acid system is discussed.

PREPARATION OF ENZYME

Assay—The hydrase reaction was followed by the assay of malonic semialdehyde formed from acetylenemonocarboxylic acid (Reaction 3). The standard assay was conducted in a volume of 1.0 ml. containing 50 μmoles of potassium phosphate at pH 7.9, 5 μmoles of acetylenemonocarboxylic acid, and an amount of enzyme sufficient to catalyze the formation of approximately 0.3 to 2.0 μmoles of malonic semialdehyde in 10 minutes. After incubation at room temperature (23°) for 10 minutes, the reaction was terminated by the addition of 1.0 ml. of a 10 per cent solution of perchloric acid. Aliquots of the incubation mixture were diluted to 0.5 ml. with water and 0.5 ml. of the 2,4-dinitrophenylhydrazine reagent of Friedemann and Haugen (3) was added. After 5 minutes at room temperature, 4 ml. of 5 N KOH in 90 per cent ethanol[1] were added. The resulting color was determined within 1 minute after the addition of alkali by a Klett-Summerson colorimeter with a No. 54 filter. Standards containing pyruvate or the 2,4-dinitrophenylhydrazine of malonic semialdehyde were included with each series of samples to be assayed; the two compounds yielded identical extinction coefficients.

Under the conditions outlined above, hydrase activity is a linear function of both time and enzyme concentration as shown in Fig. 1.

For the determination of the Michaelis constant, K_m, for acetylenemonocarboxylic acid hydrase a similar assay was used with three modifications: the incubation was conducted at 30°, larger aliquots of the incubation mixture were employed, and samples of the incubation mixture were removed at 2-minute intervals for a total of 10 minutes for each acetylenemonocarboxylic acid concentration.

A unit of hydrase activity is defined as that amount of enzyme that catalyzes the formation of 1.0 μmole of malonic semialdehyde in 10 minutes under standard assay conditions. Specific activity is defined as the number of units of activity per mg. of protein.

Growth—The organism was obtained by the enrichment culture technique with the use of an inorganic salt medium (4) supplemented with 0.1 per cent acetylenemonocarboxylic acid. Colonies, which were isolated after purification by plating, consisted of gram-negative, motile rods with one polar flagellum and have been identified as a member of the genus *Pseudomonas* (5). Although the pseudomonad could grow on a variety of carbon sources including glucose, α-alanine, and malonic acid, the hydrase was produced only with acetylenemonocarboxylic acid as the carbon source.

The pseudomonad was maintained by biweekly transfers on

* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

[1] S. Narrod and W. B. Jakoby, in preparation.

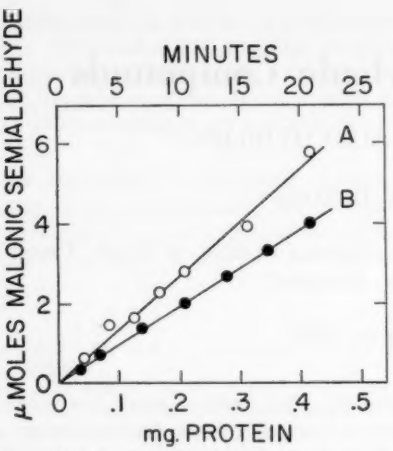


FIG. 1. Pyruvate formation under standard assay conditions as a function of time (*Curve A*) and protein concentration (*Curve B*).

TABLE I

Summary of acetylenemonocarboxylic acid hydrase purification

Fraction	Treatment	Volume	Activity	Specific activity
		ml.	*units*	*units/mg. protein*
1	Extract	71	5570	4.1
2	Protamine sulfate	92	4050*	3.6*
3	Ammonium sulfate	16.4	4700†	14†
4	Heat and dialysis	16.4	4100	38
5	Acetone	11.7	3260	49
6	Gel eluates	14	2260	175
7	DEAE-cellulose eluate‡	26	920	380

* The apparent loss of activity upon addition of protamine sulfate is presumed to reflect the inhibition by sulfate.

† The actual number of units found on assay of this fraction was 3220. This value was corrected for ammonium sulfate inhibition by multiplying by 1.47; derivation of this factor is discussed in the text.

‡ Only half of the gel eluate fraction was used for DEAE-cellulose chromatography although the values reported in the table represent the yield from the entire fraction.

agar slants containing the following per l.: K_2HPO_4, 1.15 gm.; KH_2PO_4, 0.63 gm.; NH_4NO_3, 1 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; a solution of sodium, ferrous, and manganous salts,[2] 5 ml.; previously neutralized acetylenemonocarboxylic acid, 1.5 gm.; yeast extract, 0.04 gm.; agar, 15 gm. For large scale growth experiments a similar medium was used which differed only in that yeast extract and agar were omitted.

Several milliliters of medium were used to wash organisms from a 24- to 48-hour-old agar slant. The resulting suspension served as inoculum for 1 l. of medium in a 6-l. flask. After 24 hours of shaking at approximately 28° the contents of the flask were used to inoculate three 5-gallon carboys, each of which contained 12 l. of medium and was equipped for vigorous aeration under sterile conditions. Approximately 1 gm., wet weight, of cells were obtained per l. of medium. Cells were harvested by centrifugation after 18 hours of growth at 23°. The cells were washed twice with 0.9 per cent sodium chloride and were frozen at −15°.

Extraction and Purification—Frozen cells were allowed to thaw and were suspended in 5 times their volume of 0.05 M potassium phosphate at pH 7.0 containing 5 mM mercaptoethanol. The suspension was subjected to sonic oscillation for 10 minutes in a 10 kc. Raytheon vibrator followed by centrifugation for 20 minutes at approximately 16,000 × *g*. In the preparation to be described and summarized in Table I, 13.6 gm. of cells resulted in 71 ml. of extract (Fraction 1).

To the extract were added 660 mg. of ammonium sulfate followed by 28.4 ml. of a 1 per cent solution of protamine sulfate. This operation as well as all subsequent procedures, where applicable, were conducted at approximately 0° and were accompanied by stirring. After centrifugation the residue was discarded and the supernatant fluid (Fraction 2) was treated with solid ammonium sulfate. Of ammonium sulfate 18.5 gm., 6.7 gm. and 13.6 gm. were added in sequence with centrifugation and removal of the residue after each addition. The first precipitate was discarded. The second precipitate contained an enzyme active in the oxidation of malonic semialdehyde.[3] The third precipitate which was salted out between approximately 0.4 and 0.6 of ammonium sulfate saturation, contained the hydrase and was dissolved in 0.05 M potassium phosphate at pH 7.0 which was 5 mM with respect to mercaptoethanol (Fraction 3).

Fraction 3, contained in a stainless steel vessel, was placed in a 55°-water bath and stirred. After 10 minutes of heating, the vessel was cooled in an ice bath and the precipitated protein was removed by centrifugation. The supernatant fluid (15 ml.) was dialyzed overnight against a l. of 0.05 M potassium phosphate at pH 7.0 containing 5 mM mercaptoethanol (Fraction 4).

Although only 3200 units were recovered after heating, dialysis increased this value to 4100. It is probable that the stimulation of activity was due to the removal of inhibitory sulfate ions introduced in Fraction 3. The ratio of the activity before and after dialysis, 1.43, was therefore used as a correction factor for estimating the number of units of activity present in Fraction 3 (Table I).

Precooled (−10°) acetone, 3.0 ml., was added to 16.4 ml. of Fraction 4 at 0° during a 3-minute period. The solution was transferred to a Dry Ice-ethanol bath maintained at −3° and 6.1 ml. of acetone were added. The suspension was centrifuged at −5° and the supernatant fluid was placed in a −10° bath. Acetone (29.1 ml.) was added until a final acetone concentration of 60 per cent (volume per volume) was attained. After centrifugation at −5° the residue was suspended in 10.5 ml. of 0.05 M Tris[4] buffer at pH 7.2 containing 2.5 mM mercaptoethanol. The suspension was cleared by the addition of 1 ml. of calcium phosphate gel containing 21 mg., dry weight, per ml. The supernatant fluid obtained by centrifugation is designated Fraction 5 (Table I).

A suspension of calcium phosphate gel containing 525 mg., dry weight, of solids was centrifuged and the suspending medium was discarded. The residue was thoroughly suspended in 11.7 ml. of Fraction 5 and the mixture was centrifuged; the residue was discarded. The supernatant fluid was acidified with N acetic acid to pH 6.0 and subjected to another treatment with 525

[2] Salt solution contains the following in grams per liter: NaCl, 2; $FeSO_4 \cdot 7H_2O$, 2; $MnSO_4 \cdot 4H_2O$, 2; ascorbic acid, 1.

[3] W. B. Jakoby and E. W. Yamada, in preparation.

[4] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

mg. of gel followed by centrifugation. The supernatant fluid was discarded and the gel was washed with 12 ml. of 0.05 M Tris buffer at pH 7.2 containing 2.5 mM mercaptoethanol. The gel was eluted by thorough suspension in 7 ml. of 0.05 M potassium phosphate at pH 6.7 which was 2.5 mM with respect to mercaptoethanol. The elution was repeated with 7 ml. of the same buffer and the eluates were combined (Fraction 6). The gel eluates generally yielded 50 per cent of the activity present in the crude extracts at a purification of approximately 50-fold.

Further purification could be obtained by chromatographing Fraction 6, on DEAE-cellulose (6) used as the solid phase. Certain variations of the original method (6) were used and have been described in detail previously (2). Columns of DEAE-cellulose (3 × 1 cm.) were used with 200 ml. of 0.03 M Tris buffer at pH 7.2 in the mixing flask. An ionic strength gradient was produced by allowing the same buffer, brought to 0.5 M with respect to NaCl, to flow into the mixing chamber. Of the 1200 units of activity which were absorbed, a total of 600 units was recovered in the pattern diagrammed in Fig. 2. However, only those fractions with a specific gravity greater than 300 were pooled and constitute Fraction 7. This step represents an approximately 2-fold purification over that of the gel eluates with some fractions yielding somewhat higher activity. Attempts to concentrate the protein in Fraction 7 by ammonium sulfate treatment have resulted in only 50 per cent recovery of enzyme activity.

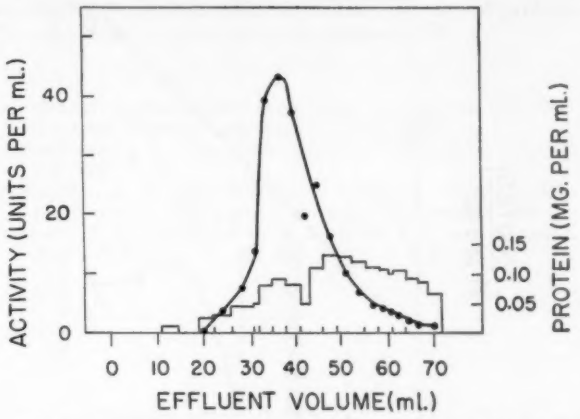


FIG. 2. Pattern of elution of the hydrase at a rate of 3 ml. per minute from a 3 × 1-cm. DEAE-cellulose column. Details are included in the text. The curve represents enzyme concentration. Protein concentration is indicated by the discontinuous line.

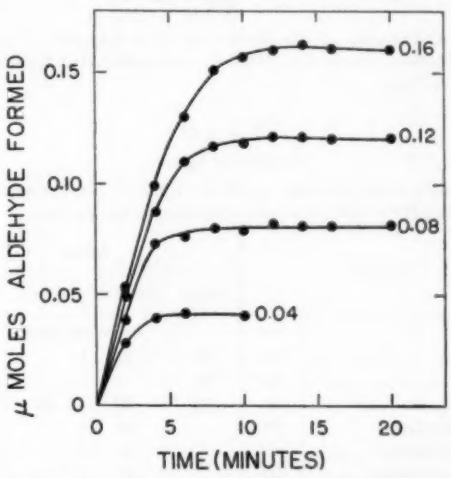


FIG. 3. Stoichiometry of the reaction. The values at the right hand side of the curves indicate the number of μmoles of acetylenemonocarboxylic acid added to the reaction mixture.

PROPERTIES OF ENZYME

Reaction Product—The product of acetylenemonocarboxylic acid hydration (Reaction 3) was identified as malonic semialdehyde by the isolation of its 2,4-dinitrophenylhydrazone. Acetylenemonocarboxylic acid, 400 μmoles, was incubated with 100 units of enzyme in 0.05 M potassium phosphate at pH 7.9 in a total volume of 20 ml. After 1 hour at room temperature the reaction was terminated by the addition of an equal volume of a 10 per cent solution of perchloric acid. Precipitated protein was removed, an excess of an acid solution of 2,4-dinitrophenylhydrazine was added and, after overnight storage at 2°, the hydrazone was separated by filtration with suction. The melting point of this compound after two recrystallizations from aqueous ethanol was found to be 159–160° (uncorrected); the 2,4-dinitrophenylhydrazone isolated from chemically synthesized malonic semialdehyde, melted over the same temperature range.

Ascending paper chromatography (butanol-water-ammonia, 100:18:1.2) of the hydrazone derivative isolated in this manner revealed one distinct spot at R_F 0.89 which was identical to that of the 2,4-dinitrophenylhydrazone prepared from synthetic malonic semialdehyde.

Pyruvic acid was not formed by the hydrase reaction as demonstrated by spectrophotometric assay with lactic dehydrogenase and DPNH. The possible addition to acetylenemonocarboxylic acid of a molecule other than water, *e.g.* NH_3, was considered. When a standard incubation mixture, including 5 units of enzyme, was made 0.2 M with respect to ammonium acetate and the products of the reaction were chromatographed, no evidence was found for the formation of ninhydrin-reacting compounds. Particular attention was directed to the possibility of α- or β-alanine formation; neither compound was detected, although malonic semialdehyde was found.

Specificity and Stoichiometry—Of the several compounds examined only acetylenemonocarboxylic acid was hydrated to form an aldehyde. Acetylenedicarboxylic acid, propynol and ethynylbenzene did not serve as substrates. The enzymatic hydration of acetylenedicarboxylic acid with the concomitant formation of malonic semialdehyde required no exogenous cofactors and occurred equally as well in a helium atmosphere as in air. As shown in Fig. 3, 1 mole of malonic semialdehyde is formed per mole of acetylenemonocarboxylic acid added.

Kinetics—A plot of enzyme activity against substrate concentration is reproduced in *Curve A* of Fig. 4. Because of inhibition by higher concentrations of substrate, 5 μmoles of acetylenemonocarboxylate acid per ml. were used in the standard assay system. The K_m for acetylenemonocarboxylic acid, as derived from initial reaction rates (Fig. 4, *Curve B*), was found to be 8×10^{-5} M at pH 8.0. The apparent energy of activation at the same pH was calculated from the initial reaction rates at four temperatures (17, 24.2, 29.6 and 37.2°) to be 7 kilocalories per mole.

Inhibition—Of the several anions investigated, only sulfate,

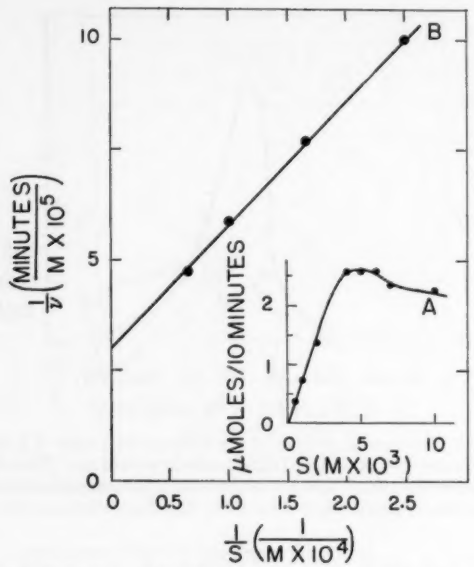


FIG. 4. Velocity of the hydration as a function of substrate concentration (*Curve A*). The K_m was estimated from *Curve B* as 8×10^{-5} M.

TABLE II

Inhibition by salts

Standard assay system at pH 8. Each of the amines was adjusted to pH 8.0 with the appropriate acid before assay.

Cations		Anions			
		Sulfate	Chloride	Nitrate	Acetate
	mM	*per cent inhibition*			
Sodium	10	45	17		0
Lithium	10	47			
Ammonium	10	50	20	25	0
Ethanolamine	50		58		36
Diethanolamine	50		60		12
Triethanolamine	50		42		0
Tris	50		90		71

chloride, and nitrate were found to be inhibitory (Table II). Thus phosphate, arsenate, acetate, malonate, succinate, carbonate, or pyrophosphate at the same concentration (10 μM) did not inhibit. Similarly, certain cations, Tris, ethanolamine, and diethanolamine, also inhibited activity whereas triethanolamine, histidine, glycine and ammonium salts of organic acids were inactive.

Enzyme activity was inhibited 50 per cent by sodium cyanide and *p*-chloromercuribenzoate at 4×10^{-4} M and 2×10^{-4} M, respectively. The following compounds at the concentrations noted were without significant effect on enzyme activity: sodium citrate (10 mM); 8-hydroxyquinoline (0.5 mM), ethylenediaminetetraacetic acid (3 mM); hydroxylamine (1 mM); hydrazine (1 mM); potassium arsenite (1 mM); *N*-methylmaleimide (1 mM).

Stability and pH Optimum—The hydrase was relatively stable at −15°; thawing after two weeks of storage at this temperature gave a solution which retained 90 per cent of the original activity. When dilute enzyme solutions were incubated at 3° for 18 hours, 20 per cent of the activity was lost over a range of pH from 7 to 8 (Fig. 5). Enzyme activity was optimal at pH 8.0 (Fig. 5).

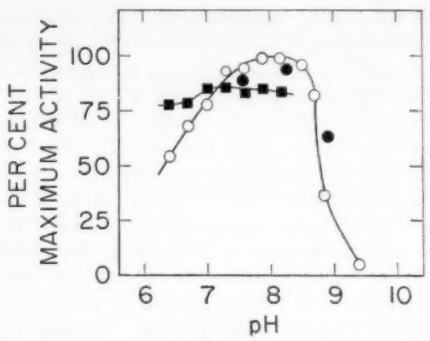


FIG. 5. The effect of pH on hydrase activity (circles) and hydrase stability (squares). For determination of the pH-activity curve 0.05 M buffers were used: phosphate (○); borate (●). The pH of each reaction mixture was determined on a duplicate sample after the 10-minute incubation period. For stability determinations, the enzyme solutions were incubated over night at 2° in 0.02 M potassium phosphate buffer. All samples were assayed at pH 7.6 at a final phosphate concentration of 0.2 M.

DISCUSSION

The hydration of acetylenemonocarboxylic acid could result in the formation of two products, pyruvic acid and malonic semialdehyde. However, it is difficult to conceive of the addition of water to an α,β-acetylenic acid with the formation of a carbonyl group at any but the β position. The literature on hydration of these compounds is amply documented with respect to the β addition of the negatively charged ion (7). It is therefore to be expected that malonic semialdehyde should be and, indeed, was isolated as the product of the enzymatic hydration of acetylenemonocarboxylic acid.

By analogy the formation of pyruvic acid from acetylenedicarboxylic acid would take place by the mechanism outlined in Reaction 1 rather than Reaction 2. Although different enzymes are involved the hydration mechanism with acetylenedicarboxylic acid may be similar to that for the hydration of acetylenemonocarboxylic acid. The scheme outlined in Reaction 1 is consistent with this interpretation. Reaction 2 supposes an initial decarboxylation to give an enzyme-acetylenemonocarboxylic acid intermediate which, in turn, would be expected to yield malonic semialdehyde rather than the observed product, pyruvic acid.

MATERIALS AND METHODS

The acetal of malonic semialdehyde ethyl ester was a gift from Dr. M. J. Coon and Dr. W. G. Robinson. The free aldehyde was prepared as suggested by Dr. Coon (personal communication). All other compounds are commercially available.

Protein was determined by the method of Lowry *et al.* (8) with crystalline bovine serum albumin used as a standard. Chromatograms were developed on Whatman No. 1 paper. When evidence for amino acid formation was sought, butanol-water-glacial acetic acid (4:1:1) was used as the developing solvent.

SUMMARY

An enzyme, obtained from extracts of a species of *Pseudomonas*, was active in the hydration of acetylenemonocarboxylic

acid (propiolic acid) with the formation of malonic semialdehyde. Purified preparations catalyze the hydration of 400 μmoles of acetylenemonocarboxylic acid per minute per mg. of protein. The mechanism of the hydration is discussed.

REFERENCES

1. Yamada, E. W., and Jakoby, W. B., *J. Am. Chem. Soc.*, **80**, 2343 (1958).
2. Yamada, E. W., and Jakoby, W. B., *J. Biol. Chem.*, **233**, 706 (1958).
3. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
4. Jakoby, W. B., *J. Biol. Chem.*, **232**, 75 (1958).
5. Breed, R. S., Murray, E. G. D., and Smith, N. R., *Bergey's manual of determinative bacteriology*, 7th edition, The Williams and Wilkins Company, Baltimore, 1957.
6. Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 756 (1956).
7. Johnson, A. W., *The chemistry of acetylenic compounds, Vol. II*, Longmans, Green and Company, Ltd., London, 1950.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall R. J., *J. Biol. Chem.*, **193**, 265 (1951).

Urinary Metabolites of Coumarin and *o*-Coumaric Acid

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Coumarin is a naturally occurring constituent of certain forages, especially clover, and is frequently ingested by livestock. It has been widely used as a flavor ingredient in foods and drugs. Hazleton *et al.* (1) have reported recently on the oral toxicity of coumarin for rats and dogs. However, little was known concerning the metabolic fate of this compound in the animal body until a recent report by Mead *et al.* (2) appeared. These workers observed hydroxylation and conjugation of coumarin when it was given orally to rats and rabbits. They found no evidence of ring fission (opening of the heterocyclic ring). In their studies approximately 75 per cent of the dose of coumarin was unaccounted for.

The investigations here reported clearly indicate that opening of the heterocyclic ring of coumarin takes place. Evidence of a species difference between rats and rabbits as regards the hydroxylation of coumarin is also presented.

EXPERIMENTAL

Materials and Methods

Coumarin, 4-hydroxycoumarin, and 7-hydroxycoumarin were purchased from commercial sources. A sample of *o*-hydroxyphenylacetic acid was obtained from Cutter Laboratories. Melilotic acid (*o*-hydroxyphenylpropionic acid) was prepared by hydrogenation of *o*-coumaric acid with palladium as a catalyst. Coumarin was converted to *o*-hydroxy-*trans*-cinnamic acid (*o*-coumaric acid) by shaking with dilute alkali and freshly precipitated mercuric oxide, as described by Seshadri (3). The glycine conjugate of *o*-coumaric acid (*o*-coumaroylglycine) was prepared from acetyl coumaroyl chloride, glycine, and sodium carbonate according to the Schotten-Baumann procedure, followed by gentle hydrolysis. Hydrogenation of *o*-coumaroylglycine yielded the glycine conjugate of melilotic acid (melilotoylglycine). The synthesis of 3-hydroxycoumarin was accomplished by means of a procedure described by Offe and Jatzkewitz (4). A sample of *o*-hydroxyphenyllactic acid was obtained by hydrolyzing 3-hydroxycoumarin with alkali, reducing with sodium amalgam, and isolating as the calcium salt from water. All compounds were examined for homogeneity by means of the two-dimensional paper chromatographic system shown in Fig. 1.

The procedures used for the collection of urine and for the extraction and chromatographic separation of its components have been described (5). The rats were maintained on a purified diet containing the following ingredients (in per cent): sucrose 71, vitamin-free casein 18, acetoolein 5, salts (U.S.P. XIV) 4, and a complete vitamin mixture in dextrose 2. The basic diet for the rabbits consisted of corn meal 49, sucrose 10, crude casein 25, corn oil 4, dried brewers' yeast 5, salts 4, magnesium oxide 0.5, and potassium acetate 2.5.

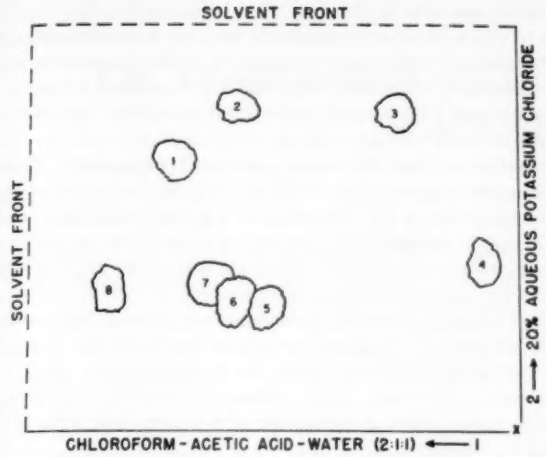
RESULTS

Rat Experiments—When adult rats were given 100 mg. of coumarin per rat (dissolved in dilute ethanol) by stomach tube, there were at least five major areas appearing on the chromatogram which were not present when control urine was similarly treated. Three of these areas were readily detected and identified as melilotic, *o*-hydroxyphenylacetic, and *o*-coumaric acids, (Areas 1, 2, and 5, respectively, Fig. 1). Identification was based on the similarities between R_F values, behavior under ultraviolet light, and color after spraying with diazotized sulfanilic acid (6), when the unknowns were compared with the authentic compounds. Additional evidence for the identity of *o*-hydroxyphenylacetic acid was based on a comparison of the crystallographic properties of an authentic sample with the material isolated from the urine of rabbits which had received coumarin. The isolation procedure is described later.

An unidentified metabolite of coumarin which gave a blue fluorescence but no color when sprayed with diazotized sulfanilic acid was observed completely surrounding the melilotic acid spot (Area 1).

The fifth spot, Area 3, Fig. 1, contained at least three substances, as became evident after acid treatment (2 N HCl reflux for 1 hour) of the corresponding area cut from an unsprayed chromatogram. Two-dimensional migration of the ether extract of the resulting hydrolysate now produced two spots in addition to a small amount of unchanged material remaining at Area 3. One of the spots corresponded to *o*-coumaric acid (Area 5); the other was identical in behavior with Area 1, corresponding to melilotic acid. The unchanged material was tentatively identified as *o*-hydroxyphenyllactic acid (see under "Rabbit Experiments"). When an unsprayed chromatogram of the ether extract from urine of rats which had received coumarin was set aside for a few weeks, Areas 1 and 3 darkened, probably as a result of oxidative deterioration. A similar darkening also occurs when melilotic acid and melilotoylglycine are treated in this manner.

The failure to detect glucuronic acid in the hydrolysate of Area 3, suggested the possibility, based on previous work (7), that *o*-hydroxyphenylhydracrylic acid might be the precursor of the *o*-coumaric acid. Hydrogenation of 4-hydroxycoumarin with sodium amalgam (with the use of CO_2 to control pH) yielded a product whose chromatographic properties were identical with



Area No.	Compound	Appearance under ultraviolet light	Color with diazotized sulfanilic acid
1	(a) Melilotic Acid	Absorbs	Orange
	(b) Unknown	Blue fluorescence	Colorless
2	*o*-Hydroxyphenylacetic acid	Absorbs	Orange
3	(a) Melilotoylglycine	Absorbs	Orange
	(b) *o*-Hydroxyphenylhydracrylic acid	Absorbs	Orange
	(c) *o*-Hydroxyphenyllactic acid	Absorbs	Orange
4	Coumaroylglycine	Tan fluorescence	Orange
5	*o*-Coumaric Acid	Tan fluorescence	Orange
6	7-Hydroxycoumarin	Blue fluorescence	Darkens
7	4-Hydroxycoumarin	Absorbs	Yellow
8	3-Hydroxycoumarin	Absorbs	Yellow

FIG. 1. Two-dimensional schematic chromatogram of urinary metabolites of coumarin.

those of Area 3. Acid treatment of this compound (2 N HCl reflux for 10 minutes) led to the formation of *o*-coumaric acid.

Rats, each of which received 100 mg. of *o*-coumaric acid by stomach tube, excreted melilotic acid, *o*-hydroxyphenylacetic acid, *o*-hydroxyphenylhydracrylic acid, melilotoylglycine, and unchanged *o*-coumaric acid in the urine. The unidentified fluorescent component surrounding the melilotic acid (Area 1) observed when rats were given coumarin, was not present when *o*-coumaric acid was given instead. An additional spot (Area 4, Figure 1) was detected after the ingestion of *o*-coumaric acid. After hydrolysis of this area, the ether extract of the hydrolysate was chromatographed and *o*-coumaric acid (Area 5) was identified. The substance in Area 4 was found to have the same chromatographic properties as synthetic *o*-coumaroylglycine.

When melilotic acid was given to rats (100 mg. each) by stomach tube the urinary metabolites excreted in the urine were identical with those excreted when *o*-coumaric acid was given. In Area 7, Fig. 1, a substance corresponding to 4-hydroxycoumarin was found to be present in slight amounts not only when rats were given melilotic acid, but also when either coumarin or *o*-coumaric acid was given.

Finally, rats were given the calcium salt of *o*-hydroxyphenyllactic acid by stomach tube (100 mg. each). The only detectable metabolite on the chromatogram was *o*-hydroxyphenylacetic acid. Unchanged *o*-hydroxyphenyllactic acid was also present.

Estimates of the amounts of urinary metabolites excreted by rats were based on comparisons of spot sizes on chromatograms containing appropriate volumes of urine with known amounts (5 to 10 μg.) of the authentic compounds. Of a total dose of 200 mg. of coumarin 10 to 15 mg. of *o*-hydroxyphenylacetic acid and 5 to 7 mg. of melilotic acid were accounted for in the urine. A minimum of 4 to 6 mg. of *o*-coumaric acid was detected in the urine after acid hydrolysis, which included the *o*-coumaric acid obtained by conversion of *o*-hydroxyphenylhydracrylic acid to *o*-coumaric acid.

Rabbit Experiments—Rabbits were allowed to adjust to the semipurified diet for several days before the addition of coumarin at a level of 0.5 per cent. Urine was collected, acidified, subjected to extraction with ether, and chromatographed in the same manner as described for rat urine. A major metabolite was *o*-hydroxyphenylacetic acid the identity of which was confirmed by crystallographic comparison of the isolated material with an authentic sample. Melilotic acid was also present surrounded by the unidentified blue fluorescing material previously observed in rat urine. Very little *o*-coumaric acid was detected until after Area 3 was refluxed with acid, suggesting that *o*-hydroxyphenylhydracrylic acid was the precursor. Also present on the chromatogram of the ether extract of Area 3 was melilotic acid, indicating the presence of melilotoylglycine at Area 3. Some of the material at Area 3 was resistant to acid hydrolysis (2 N HCl reflux for 1 hour). Synthetic *o*-hydroxyphenyllactic acid was found to be stable to acid hydrolysis and had the same R_f values and color with diazotized sulfanilic acid as this acid resistant urinary component.

Relatively large amounts of two metabolites not detected in rat urine were 3-hydroxycoumarin and 7-hydroxycoumarin (Areas 8 and 6, respectively, Fig. 1). Identification of the two hydroxylated derivatives of coumarin was based not only on paper chromatographic properties but also on the crystallographic properties following their isolation from urine.

The isolation of *o*-hydroxyphenylacetic acid, 3-hydroxycoumarin, and 7-hydroxycoumarin was accomplished by Soxhlet extraction with alcohol of lyophilized unacidified urine from rabbits receiving coumarin orally. The alcohol was removed by

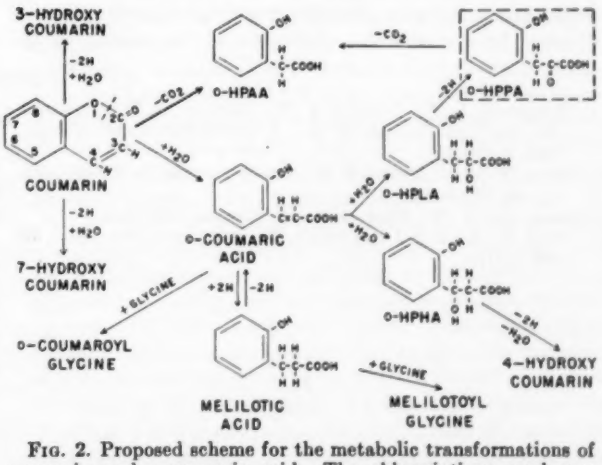


FIG. 2. Proposed scheme for the metabolic transformations of coumarin and *o*-coumaric acid. The abbreviations used are: o-HPAA, *o*-hydroxyphenylacetic acid; o-HPPA, *o*-hydroxyphenylpyruvic acid; o-HPLA, *o*-hydroxyphenyllactic acid; and o-HPHA, *o*-hydroxyphenylhydracrylic acid.

evaporation and the residue dissolved in aqueous HCl; this solution was subjected to extraction successively with carbon tetrachloride and chloroform. The carbon tetrachloride solution was decolorized with charcoal and the 3-hydroxycoumarin and 7-hydroxycoumarin were separated by fractional crystallization from water. The chloroform extract which contained the *o*-hydroxyphenylacetic acid was chromatographed on a cellulose column with the use of chloroform-acetic acid-water (2:1:1). The *o*-hydroxyphenylacetic acid was crystallized from the appropriate cellulose column eluate.

When rabbits received *o*-coumaric acid in the diet (1 per cent) an examination of the urine by means of paper chromatography revealed results almost identical to those obtained for the rat. Thus, in addition to the compound fed, melilotic acid, *o*-coumaroylglycine, melilotoylglycine, 4-hydroxycoumarin, *o*-hydroxyphenylhydracrylic acid, and *o*-hydroxyphenylacetic acid were detected in the urine. Neither 3-hydroxycoumarin nor 7-hydroxycoumarin was present.

DISCUSSION

In Fig. 2 we have attempted to summarize the relationships of the various urinary metabolites arising as a result of the opening of the heterocyclic ring of coumarin, as well as hydroxylation, decarboxylation, and conjugation of coumarin. With the exception of *o*-hydroxyphenylpyruvic acid, all compounds shown in Fig. 2 were either isolated or detected chromatographically, or both. Formation of *o*-hydroxyphenylacetic acid is shown to take place by two routes. The major route proceeds directly from coumarin since greater amounts of *o*-hydroxyphenylacetic acid were excreted by both rats and rabbits after ingestion of coumarin than when *o*-coumaric acid was fed. Intermediates, if any exist, have not been identified for this pathway. A second route leading to the formation of *o*-hydroxyphenylacetic acid from *o*-coumaric acid via *o*-hydroxyphenyllactic acid and *o*-hydroxyphenylpyruvic acid is equally certain. The possibility that *o*-coumaric acid was converted to *o*-hydroxyphenylacetic acid by way of coumarin can be ruled out since rabbits receiving *o*-coumaric acid did not excrete any 3-hydroxycoumarin or 7-hydroxycoumarin, whereas large amounts of these two compounds were found in the urine of rabbits receiving coumarin. Further evidence for the secondary pathway was the presence of appreciable amounts of *o*-hydroxyphenylacetic acid in the urine of rats to which *o*-hydroxyphenyllactic acid had been administered.

Our findings pertaining to the opening of the heterocyclic ring of coumarin in the animal body and the absence of 3-hydroxycoumarin and 7-hydroxycoumarin in rat urine after ingestion of coumarin are at variance with the report of Mead *et al.* (2). An explanation of these differences is not readily apparent. These workers also suggested that *o*-hydroxyphenylhydracrylic acid was an intermediate in the formation of 4-hydroxycoumarin. Our work clearly indicates the presence of appreciable amounts of *o*-hydroxyphenylhydracrylic acid in rat and rabbit urine as well as smaller amounts of 4-hydroxycoumarin.

The formation of *o*-hydroxyphenylhydracrylic acid from *o*-coumaric acid may be visualized to proceed simply by the addition of water across the double bond, the hydroxyl group going on the β carbon of the side chain. However, if the hydroxyl group goes on the α carbon of *o*-coumaric acid a lactic acid derivative would be formed.

The metabolism of *m*- and *p*-coumaric acids has already been shown to proceed via β oxidation to produce the corresponding benzoic acid derivatives (7, 8). The absence of salicylic acid in the urine after the ingestion of *o*-coumaric acid suggests that the presence of a hydroxyl group *ortho* to the 3 carbon side chain may either inhibit β oxidation, or favor decarboxylation to yield *o*-hydroxyphenylacetic acid. Mead *et al.* (2) also reported the absence of salicylic acid as a metabolite of coumarin.

The interconversion of *o*-coumaric acid and melilotic acid appears to take place readily. A similar condition has been observed for ferulic and dihydroferulic acids (5).

SUMMARY

The finding of *o*-hydroxyphenylacetic acid in the urine of rats or rabbits receiving coumarin orally indicated that opening of the lactone ring and decarboxylation had occurred, though by an undetermined route. An alternate pathway for the formation of *o*-hydroxyphenylacetic acid involves *o*-coumaric acid and *o*-hydroxyphenyllactic acid as intermediates. Some of the *o*-coumaric acid was converted to *o*-hydroxyphenylhydracrylic acid and 4-hydroxycoumarin. A species difference was encountered in that rabbits excreted 3-hydroxycoumarin and 7-hydroxycoumarin after ingestion of coumarin whereas rats did not excrete either of these compounds in detectable amounts.

REFERENCES

1. Hazleton, L. W., Tusing, T. W., Zeitlin, B. R., Thiessen, R., Jr., and Murer, H. K., *J. Pharmacol. Exptl. Therap.*, **116**, 348 (1956).
2. Mead, J. A. R., Smith, J. N., and Williams, R. T., *Biochem. J.*, **68**, 67 (1958).
3. Seshadri, T. R., *Chem. and Ind. London*, 308 (1954).
4. Offe, H. A., and Jatzkewitz, H., *Ber. deut. chem. Ges.*, **80**, 469 (1947).
5. Booth, A. N., Emerson, O. H., Jones, F. T., and DeEds, F., *J. Biol. Chem.*, **229**, 51 (1957).
6. Bray, H. G., Thorpe, W. V., and White, K., *Biochem. J.*, **46**, 271 (1950).
7. Booth, A. N., Jones, F. T., and DeEds, F., *J. Biol. Chem.*, **230**, 661 (1958).
8. Booth, A. N., Jones, F. T., and DeEds, F., *J. Biol. Chem.*, **233**, 280 (1958).

The Desulfuration of Thiourea by Thyroid Cytoplasmic Particulate Fractions*

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Thiourea, a goitrogen (1, 2) and an inhibitor of the iodination of tyrosyl groups in the thyroid (3), is itself metabolized by this organ. The sulfur of thiourea is converted largely to inorganic sulfate (4, 5) and partially to protein-bound sulfur and two unidentified sulfur compounds (5). Recently thiosulfate[1] has also been found among the products of the metabolism *in vivo* of thiourea by the rat thyroid. This report will describe a cytoplasmic particulate system *in vitro* from mammalian thyroid which desulfurates thiourea to sulfur-containing products similar to those observed *in vivo*.

EXPERIMENTAL

Materials—S^{35}-thiourea, C^{14}-thiourea, and S^{35}-thiocyanate were obtained from the New England Nuclear Corporation; the specific activities ranged from 15.0 to 40.0 mc. per mmole. The chromatographically pure compounds were dissolved in triple-distilled water, and the solutions were stored at −20°. S^{35}-sulfide in 0.02 N NaOH, with a specific activity of 4.2 mcuries per mmole, was obtained from the same source.

Dehydroascorbic acid, potassium selenocyanate, and 3,4-dichlorophenylserine were kindly furnished by Drs. P. L. Munson, J. Wolff, and D. Gilbert, respectively. Thyroglobulin (Preparation CH-29, 91921 A) was supplied by Dr. R. L. Kroc of the Warner-Chilcott Laboratories.

DPNH and TPNH were obtained from the Sigma Chemical Company. Wherever necessary, compounds were neutralized with potassium hydroxide before use.

Preparation of Thyroid Cytoplasmic Particulate Fractions—Thyroids from freshly killed sheep were obtained at local abattoirs, chilled on ice, and cleaned of extraneous matter. The tissue was minced with a razor blade and disintegrated with 9 volumes of 0.25 M sucrose-0.027 M $KHCO_3$ in a chilled Servall Omni-Mixer run at half speed for 15 seconds. The mixture was ground gently in a chilled homogenizer of the Potter-Elvehjem type until a homogeneous suspension resulted. Unbroken cells, nuclei, and tissue debris were removed by two successive centrifugations in a refrigerated International Centrifuge at 600 × *g* for 5 minutes. The supernatant fluid (I) was then centrifuged for 10 minutes at 8,500 × *g* to collect the "large particles." The supernatant fluid (II) from the latter centrifugation was centrifuged for 60 minutes at 105,000 × *g* in the No. 40 rotor of the Spinco Preparative Ultracentrifuge to collect the "small particles." The supernatant solution from this centrifugation is designated the "soluble fraction." For most of the experiments described in this paper, supernatant fluid I was centrifuged directly for 60 minutes at 105,000 × *g*, thus collecting all the cytoplasmic particles as one pellet. Pellets were suspended by homogenation in 0.1 M Tris,[2] pH 7.4, containing 0.25 M sucrose or 0.15 M KCl. A ml. of this suspension contained the particles from 1.0 gm. of thyroid tissue.

Liver and Kidney Particulate Fractions—Male rats weighing approximately 150 gm. were decapitated. The liver and kidneys were excised rapidly, minced, and washed free of red blood cells and urine by suspending the tissue in a chilled solution of 0.25 M sucrose-0.027 M $KHCO_3$. Homogenation and differential centrifugation were carried out as above.

Incubation—The incubation mixture contained the tissue fraction, S^{35}- or C^{14}-thiourea (approximately 125,000 c.p.m. in 50 mμmoles), thiocyanate, ascorbic acid or dehydroascorbic acid, and Tris buffer, pH 7.4. The flasks were shaken in a water bath at 37° for 1 hour. The reaction was stopped by placing the flasks on ice.

Analytical Methods—The S^{35}-metabolites were characterized by paper chromatography. A 20-μl. aliquot of the incubation mixture and 0.2 μmole each of thiourea, thiosulfate, and of thiocyanate carriers were applied along a 1.5-cm. line to Whatman No. 1 chromatography paper. Chromatograms were run in an ascending solvent system of ethanol-ammonium acetate (1 M), pH 7.5 (7.5:3). The radioactive spots along the paper strips were located by radioautography with the use of No-Screen x-ray film. The carrier spots were identified as follows: thiourea by ultraviolet quenching; and thiosulfate and thiocyanate by spraying with 0.1 N iodine and 10 per cent ferric chloride, respectively. A typical chromatogram is shown in Fig. 1*A*. The percentage of each product formed and of thiourea converted was measured by cutting up the chromatogram and assaying the radioactivity in each area as a percentage of the total number of counts along the strip. Blank values were obtained for each experiment from

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[1] F. Maloof, unpublished observations.

[2] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

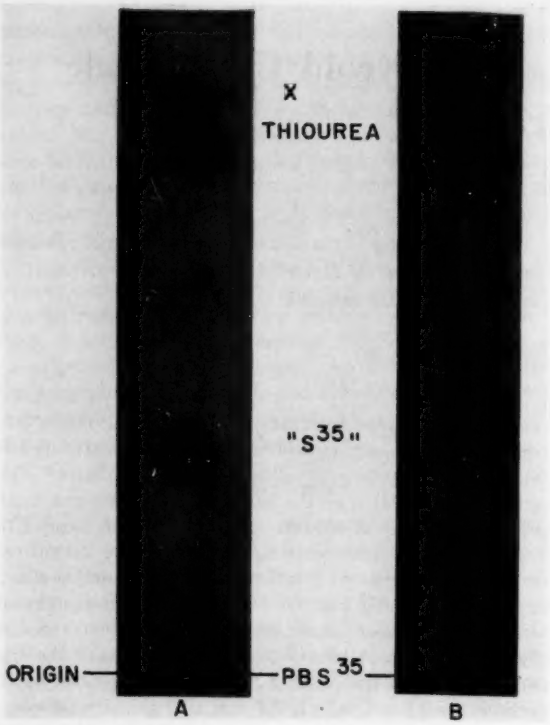

FIG. 1. Radioautogram of paper chromatogram run in an ascending solvent system of ethanol-1.0 M ammonium acetate, pH 7.5 (7.5:3). *A*, represents the results obtained after incubation as described in the legend of Table I; *B*, a control, heated at 100° for 1 minute. This solvent separates a radioactive spot which remains at the origin (protein-bound S^{35}, PBS^{35}), an inorganic S^{35} spot with an R_F of 0.25 which is a mixture of thiosulfate and sulfate, a thiourea spot with an R_F of 0.77, and an unknown spot with an R_F of 0.84. The R_F of thiocyanate in this solvent is 0.92.

TABLE I

Requirements for desulfuration of thiourea by thyroid cytoplasmic particles

The complete system contained the thyroid cytoplasmic particles (1.5 mg. of protein), 10^{-3} M KSCN, 10^{-3} M ascorbic acid, 5×10^{-5} M S^{35}-thiourea, 0.15 M Tris buffer, pH 7.4, in a final volume of 1.0 ml. Incubation, 1 hour at 37°.

System	Thiourea desulfurated
	mμmoles
Complete system	12.0
Complete system minus ascorbic acid	1.0
Complete system minus thiocyanate	0.8
Complete system minus particulate fraction	0.5
Complete system (with heated particulate fraction)	1.0

a heated control (Fig. 1*B*). The S^{35} radioactivity was counted by a thin end window Geiger-Mueller tube (window thickness, 0.9 mg. per cm.[2]).

Protein analyses were done by means of the biuret (6) and the micro-Kjeldahl methods (7). Iodine was determined by a modification of Chaney's method (8).

The amount of thiourea desulfurated varied from one preparation to another. However, since the particulate preparation was stable at 4° for 2 weeks, a number of experiments could be carried out with the same material. All experiments were performed at least two or three times. Each table represents a typical run.

RESULTS

In Table I are set out the requirements for the desulfuration of thiourea by cytoplasmic particles from sheep thyroid. It is clear that, in addition to the protein, there is a strict requirement for thiocyanate and ascorbic acid.

Characterization of Thyroid Cytoplasmic Particles—The "large particles" had succinoxidase activity[3] comparable with that of an equal weight of liver mitochondria; the "small particles" showed no measurable succinoxidase activity.

Ultracentrifugal analysis[4] of a twice-washed pellet of "small particles" in a medium of 0.25 M sucrose-0.1 M glycylglycine, pH 7.6, revealed the fraction to be mainly heterogeneous except for a small, sharp peak, $s_{20,w}$ = 51, probably ribonucleoprotein particles (9, 10). The protein-bound iodine content of this fraction was 0.12 per cent. If this represents thyroglobulin iodine, then thyroglobulin (approximate iodine content, 0.5 to 1.0 per cent) is only 10 to 20 per cent of the total protein of the "small particles." An alternative explanation is that this fraction contains an incompletely iodinated protein, possibly a precursor of thyroglobulin. Electron microscopy[5] (RCA model EMU 3B) of a twice-washed pellet of "small particles," fixed in osmium tetroxide, revealed a heterogeneous mass of components which resembled previously described microsomes (11). Electron microscopy of the "large particles" revealed the presence of mitochondria plus components similar to those seen in the sections of "small particles."

Activity of Various Cytoplasmic Particles of Thyroid—The combined "large plus small particles" of sheep thyroid were used in most experiments, since, as can be seen from Table II, these two fractions possess approximately equal activities. The inability of the whole homogenate to desulfurate thiourea is due to an inhibition by the soluble fraction. This, however, is not a specific property of the thyroid soluble fraction, since the corresponding fraction of rat liver is equally inhibitory. In both instances, coagulation of the soluble proteins destroyed the inhibition.

Although all fractions contain iodine, there is no clearly perceptible relationship between iodine content and activity.

Properties of Thyroid Cytoplasmic Particulate Fraction—The system is heat-labile, 1 minute at 100° sufficing to abolish all activity. While it is stable at 4° for at least 2 weeks, there is a 40 per cent loss of activity after 7 days at −20°. Dialysis of the preparation overnight at 5° results in no loss of activity.

Protein Concentration—The desulfuration of thiourea as a function of protein concentration is shown in Fig. 2.

Thiourea—The system is saturated with 40 to 50 mμmoles per ml. (Fig. 3).

Thiocyanate—At a concentration of 10^{-3} M, the system is saturated (Fig. 4). To date no substitute has been found for thiocyanate. Ions tested were cyanate, selenocyanate, methylthiocyanate, perchlorate, iodide, bromide, chloride, fluoride, benzenesulfonate, trichloroacetate, sodium and magnesium Ver-

[3] Kindly assayed by Dr. Arnold F. Brodie.

[4] We are indebted to Dr. Karl Schmid and Miss A. Polis for the ultracentrifugal analyses.

[5] Performed by Dr. David Spiro.

TABLE II

Desulfuration of thiourea by various cytoplasmic fractions of thyroid

Centrifugal fractionation as described in the "Experimental." The washing medium was 0.15 M KCl-0.1 M Tris, pH 7.4. Incubation conditions as in Table I.

Tissue fraction	Total iodine*	Thiourea desulfurated
	μg./mg. protein	mμmoles/mg. protein
Whole homogenate		0.4
Large particles	1.0	5.6
washed once	0.5	4.5
washed twice	0.3	5.2
Small particles	2.5	4.5
washed once	2.1	6.6
washed twice	1.2	8.0
Large plus small particles†		6.1
Soluble fraction	6.9	0.2
Large plus small particles plus soluble fraction‡		0.8

* This iodine is considered to be protein bound, since dialysis removed none of it.

† Washed preparations; on a weight basis, the protein in this incubation was half "large particles" and half "small particles."

‡ Similar to †, plus 1.7 mg. of soluble fraction protein.

senate, 1,10-*o*-phenanthroline, urea, cysteine, glutathione, and tyrosine.

L-Ascorbic Acid—The saturation concentration of ascorbic acid is 10^{-3} M (Fig. 5). It can be replaced by equimolar concentrations of dehydroascorbic, *d*-isoascorbic acid, or DPNH. TPNH is inactive. Hydroquinone, dichlorophenolindophenol, and methylene blue are 30 to 50 per cent effective. Cysteine and reduced glutathione are inactive. Attempts were made to replace ascorbic acid with metal ions, Cu^{+}, Fe^{++}, and Mn^{++}, but all of these salts produced a nonenzymatic desulfuration of thiourea.

Anaerobiosis—The desulfuration of thiourea was inhibited to the extent of 80 per cent by anaerobiosis.

pH Optimum—The pH optimum was found to be 7.2 to 7.4.

Kinetics—The desulfuration of thiourea has a maximal rate during the first 30 minutes (Fig. 6). This rate varied from preparation to preparation, ranging from 125 to 420 mμmoles per gm. of thyroid per hour. This approximates the rate *in vivo* in rats of about 250 mμmoles per gm. of thyroid per hour.[1]

Liver and Kidney Tissues—The particulate fractions of rat liver and kidney desulfurated only an insignificant amount of thiourea (Table III). Negative results were also obtained when thyroglobulin, β-lactoglobulin, or crystalline bovine albumin served as the protein source.

Reaction Products—S^{35}-thiourea is converted chiefly to protein-bound S^{35} (about 54 per cent of the products), thiosulfate (27 per cent), sulfate (9 per cent), and an unidentified compound (10 per cent) (Fig. 1).

Protein-bound S^{35}—The reasonable conclusion that the S^{35} is bound to protein stems from the following observations: (*a*) the radioactivity precipitates along with the protein on denaturation with trichloroacetic acid; (*b*) the radioactivity is nondialyzable; and (*c*) the radioactivity does not separate from the protein under a variety of conditions of paper chromatography and paper electrophoresis.

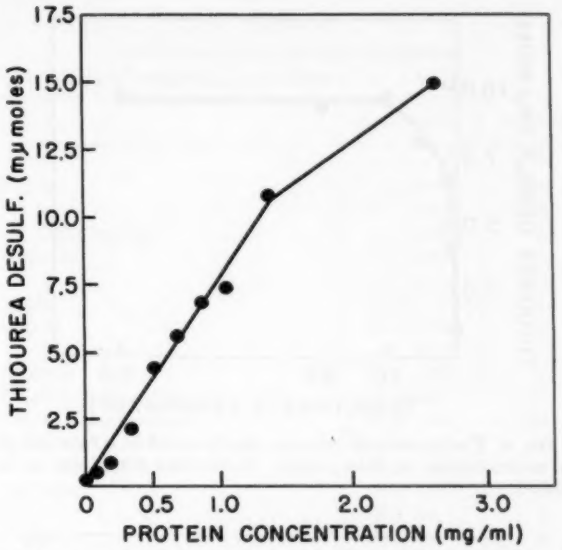

FIG. 2. The amount of thiourea desulfurated as a function of the protein concentration. The preparation was washed once in a medium of 0.15 M KCl-0.1 M Tris, pH 7.4. Incubation conditions as in Table I.

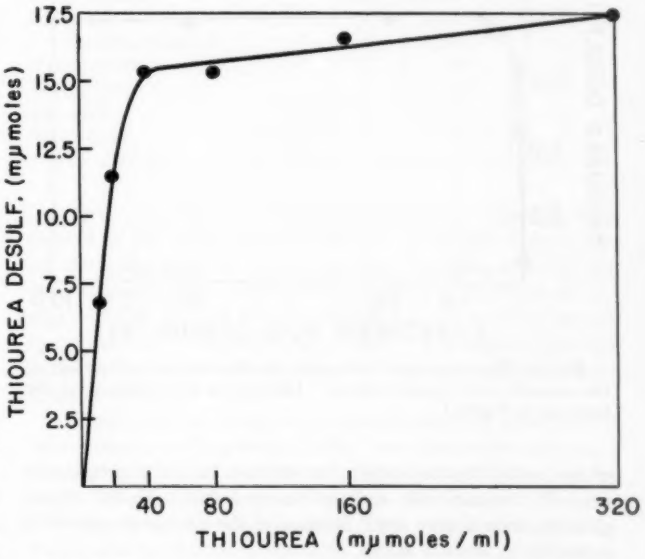

FIG. 3. The amount of thiourea desulfurated as a function of the substrate concentration. Incubation conditions as in Table I.

Attempts were made to identify this protein by paper electrophoresis with the use of Whatman No. 1 paper, barbital buffer (0.12 M), ionic strength 0.1, pH 8.6, and 125–150 volts for 18 hours. The paper was dried in an oven for 30 minutes at 100° and then stained with Amido Schwartz (Bender & Hobein, Munich, Germany). The protein-bound S^{35} does not move in this electrophoretic system, in contrast to thyroglobulin which travels between serum α_1-globulin and albumin. Solubilizing the protein-bound S^{35} with sodium desoxycholate (1 or 4 per cent) at 37° for 2 hours led to the movement of only a small portion of the protein along with its radioactivity. The mobility

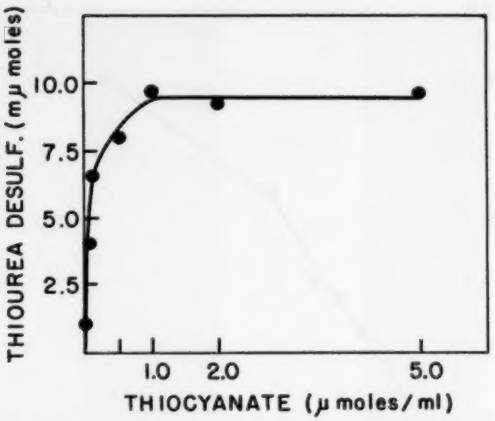

FIG. 4. The amount of thiourea desulfurated as a function of the concentration of thiocyanate. Incubation conditions as in Table I.

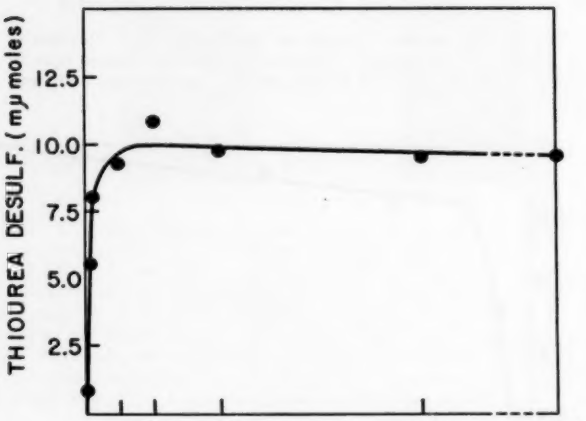

FIG. 5. The amount of thiourea desulfurated as a function of the ascorbic acid concentration. Incubation conditions were the same as in Table I.

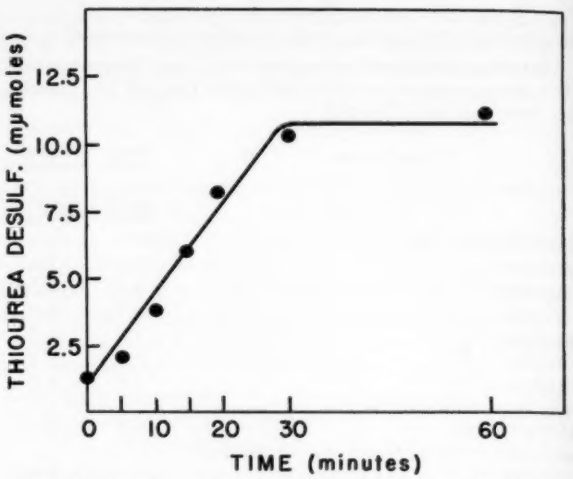

FIG. 6. The amount of thiourea desulfurated as a function of time. Incubation conditions as in Table I, except that dehydroascorbic acid (10^{-3} M) was used in place of ascorbic acid.

TABLE III

Desulfuration of thiourea by cytoplasmic particulate fractions of sheep thyroid and rat liver and kidney

Centrifugal fractionation as described in the "Experimental." Incubation conditions as in Table I.

Tissue particles	Thiourea desulfurated
	mμmoles/mg. protein
Thyroid, large plus small particles	5.9
Liver, large plus small particles*	0.6
Kidney, large plus small particles*	0.5

* Results were similar whether these fractions were tested separately or together.

of this small fraction varied, but was not that of thyroglobulin similarly treated with sodium desoxycholate. Unlike thyroglobulin, only a very small fraction of the protein-bound S^{35} is extracted by isotonic saline.

Attempts were made to determine the nature of the sulfur bound to protein. It was possible to demonstrate that the intact thiourea molecule does not become attached to protein. When C^{14}-labeled thiourea was incubated in place of the S^{35}-thiourea, no protein-bound C^{14} was found after incubations ranging from zero to 60 minutes. Hence, only the sulfur portion of thiourea becomes attached to protein during the course of the reaction. C^{14}-urea was identified as one of three C^{14}-labeled products formed during this incubation; cyanamide was not one of the products.

Dialysis of the protein-bound S^{35} for 24 hours at 4° against water, Tris buffer, or sodium chloride results in the loss of less than 5 per cent of the radioactivity. The S^{35} is not displaced by reincubation of the protein-bound S^{35} with nonradioactive thiourea (10^{-2} M), sulfide (5×10^{-3} M), or sulfate (10^{-2} M) at 37° for 30 minutes. After 2 weeks in solution at 4° 80 per cent of the radioactivity remains attached to the protein. The protein-bound S^{35} also resists the action of 1 N HCl at room temperature for 2 hours.

On the other hand, it was possible to remove the sulfur label from the protein-bound S^{35} by simple incubation of the latter mixture at room temperature for 2 hours with certain alkaline reagents, such as cyanide (0.01 M), bisulfite (0.01 M), or 0.1 N sodium hydroxide. The products were identified by paper chromatography and by the system of paper electrophoresis described below. These alkaline reagents displaced 60 to 80 per cent of the radioactivity from the protein. Thiocyanate was the major product resulting from the reaction with cyanide, while sulfite gave a good yield of thiosulfate. Sulfite was found as a product of the incubation with NaOH. These data suggest that the S^{35} exists on the protein at more than one level of oxidation. About 20 per cent of the radioactivity was not removed by these alkaline reagents.

Inorganic S^{35} Spot—The heterogeneity of the inorganic S^{35} was established by paper electrophoresis with the use of thin Whatman No. 31 paper, Tris-citrate buffer (0.06–0.03 M), ionic strength 0.18, pH 7.0, 1000 volts for 35 minutes (12). This method sep-

arates sulfite with a mobility of 15.0 cm., sulfate with a mobility of 19.0 cm., and thiosulfate with a mobility of 21.0 cm. under a potential gradient of 16.7 volts per cm. Sulfite and thiosulfate were identified by spraying the paper with 0.1 N alcoholic iodine; sulfate by using S^{35}-labeled sulfate as a marker. The inorganic S^{35} spot was found on electrophoresis to consist of thiosulfate and sulfate in the proportion of 3:1. Thiocyanate has the same mobility as sulfate in this system, but is easily separated from it by paper chromatography (Fig. 1).

Experiments with S^{35} Sulfide—The possibility was considered that the desulfuration of thiourea might result from a preliminary hydrolysis to inorganic sulfide followed by oxidation of the latter in the manner described by several investigators (13–19). To test this point, labeled sulfide (50 mμmoles) was incubated in the usual way with tissue preparations from sheep thyroid and rat liver and kidney. It was found that sulfide was transformed to approximately the same extent in all these systems to yield thiosulfate and protein-bound S^{35}. Since oxidation of sulfide, in these small amounts, occurred as well in the absence of protein, the process is clearly nonenzymatic.

These experiments, however, revealed certain important differences between the reactions of thiourea and sulfide. The latter was found to give rise to no detectable amount of sulfate, whereas thiourea formed small, but reproducible, quantities of this ion. Furthermore, the presence of mersalyl (10^{-3} M) had a profound effect on the reaction of sulfide, completely inhibiting the formation of protein-bound S^{35}, while the conversion of thiourea-sulfur to protein-bound S^{35} was barely affected (25 per cent inhibition). For these reasons, the desulfuration of thiourea is thought to proceed by way of direct transfer of sulfur to protein and not via preliminary hydrolysis to inorganic sulfide.

Inhibitors—The desulfuration of thiourea was found to be inhibited by iodide, cyanide, azide, sulfide, a number of thiol compounds, and several aromatic antithyroid substances. These and a number of other common enzyme inhibitors are listed in Table IV. Of considerable interest is the fact that, of the halides, only iodide is inhibitory, and that this inhibition is partially reversed by increasing the thiocyanate concentration. Thiosulfate is inhibitory but not at the low concentration produced in the desulfuration reaction. There is no inhibition by 3,4-dichlorophenylserine which is reported to be a specific copper-enzyme inhibitor (20).

TABLE IV

Inhibition of desulfuration of thiourea

Incubation conditions as in Table I.

	Concentration	Inhibition
	M	%
Iodide	1×10^{-3}	78
	1×10^{-4}	22
All other halides	4×10^{-3}	5
Cyanide	1×10^{-4}	91
	1×10^{-5}	30
Azide	1×10^{-3}	84
Sulfide	5×10^{-4}	82
	1×10^{-5}	4
Cysteine	1×10^{-3}	94
Diethyldithiocarbamate	1×10^{-4}	93
Glutathione (reduced)	5×10^{-4}	80
Thiosulfate	1×10^{-3}	88
	4×10^{-6}	0
Bisulfite	1×10^{-4}	92
	1×10^{-5}	6
Urea	1×10^{-3}	0
Sulfate	1×10^{-2}	14
Sulfanilamide	4×10^{-3}	46
Sulfadiazine (Na salt)	4×10^{-3}	38
p-Aminobenzoic acid	4×10^{-3}	52
Dinitrophenol	1×10^{-3}	4
Arsenite	1×10^{-3}	0
Dichlorophenylserine	1×10^{-3}	0
Hydroxylamine	1×10^{-3}	0
Antimycin A	1×10^{-4}	0
Mersalyl	1×10^{-4}	0
Perchlorate	1×10^{-3}	1

DISCUSSION

This study describes a cytoplasmic particulate system derived from thyroid tissue which is capable of desulfurating thiourea and oxidizing the sulfur to inorganic sulfate. This system is heat-labile, nondialyzable, and pH dependent. Particulate fractions from liver and kidney, prepared and incubated in a similar manner, are much less active. This difference is also apparent *in vivo*; the thyroid can metabolize a much greater quantity of thiourea than an equal weight of liver (5).

Several of the sulfur products formed in the experiments *in vitro* are the same as those found *in vivo*. The major product of the metabolism *in vivo* of thiourea in the thyroid is inorganic sulfate (4, 5), but protein-bound sulfur (5) and thiosulfate[1] are also found. The system *in vitro*, however, produces mainly protein-bound sulfur, along with some thiosulfate and sulfate.

From the experiments with labeled sulfide, it is concluded that the desulfuration of thiourea does not result from the hydrolysis to inorganic sulfide. On the contrary, it appears that sulfur is transferred directly from thiourea to thyroid protein; and only thyroid, of the tissues tested (Table III), is capable of dissociating the sulfur atom from thiourea and converting it to the inorganic state. The desulfuration reaction is inhibited by inorganic iodide and the aromatic antithyroid compounds, but not by perchlorate. This is the same pattern of inhibition observed in the iodination of tyrosyl groups by thyroid cytoplasmic particulates (21).

Astwood (22) has already demonstrated that the effective sulfur-containing goitrogens invariably have a thiocarbamide group as a feature of their molecular constitution. From the present study, the possibility emerges that it is the cleavage of the carbon-sulfur bond, by thyroid tissue, that underlies the antithyroidal activity of these compounds.

Thiocyanate is a specific requirement for the desulfuration *in vitro* of thiourea. Noteworthy is the fact that perchlorate cannot replace thiocyanate even though it is 10 times as active as an inhibitor of the concentration of iodide by the thyroid (23). Moreover, it is significant that, of the halide ions, only iodide is inhibitory; the partial reversibility of this inhibition by increasing the concentration of thiocyanate is in accord with the well established competition between these two anions in thyroid physiology (22). These facts raise again the question as to a possible role of thiocyanate in the normal metabolism of the thyroid (24).

Although it has been reported that thiocyanate is oxidized to sulfate in the rat thyroid (25), no such oxidation *in vitro*, with S^{35}-labeled thiocyanate added, was demonstrable under the present experimental conditions.

The function of ascorbic acid in the desulfuration of thiourea appears to be less specific, since it can be replaced by dehydroascorbic acid, *d*-isoascorbic acid, and DPNH. TPNH, an essential cofactor in the deiodination *in vitro* of monoiodotyrosine and diiodotyrosine by thyroidal microsomes, does not replace ascorbic acid (26, 27). The requirement for a reducing agent is reminiscent of similar requirements in the enzymatic oxidation of tyrosine (28, 29) and various drugs (30).

A considerable effort was made to identify the protein in the thyroid cytoplasmic particles to which the S^{35} becomes attached; more specifically, it seemed important to know if this protein might be thyroglobulin. From its electrophoretic and ultracentrifugal behavior, its iodine content, and its very limited extractability into isotonic saline, it appears that the major part of the protein-bound S^{35} is not sulfur-labeled thyroglobulin.

SUMMARY

A cytoplasmic particulate system, derived from sheep thyroid, has been described which desulfurates thiourea in the presence of thiocyanate and a reducing agent, such as ascorbic acid. The system is heat-labile, nondialyzable, and pH dependent. Particulate fractions from rat liver and kidney are inactive.

The sulfur products of this system *in vitro* are protein-bound sulfur, thiosulfate, and sulfate. The protein-bound sulfur is apparently not sulfur-labeled thyroglobulin.

Acknowledgments—The authors wish to thank Drs. J. B. Stanbury, O. Cope, and P. Zamecnik for their encouragement and Dr. M. Soodak for helpful discussions. We are especially indebted to Dr. Elizabeth B. Keller for invaluable technical advice. Miss Lena Neri provided technical assistance.

REFERENCES

1. MacKenzie, C. G., and MacKenzie, J. B., *Endocrinology*, **32**, 185 (1943).
2. Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, **32**, 210 (1943).
3. Keston, A. S., Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *J. Biol. Chem.*, **152**, 241 (1944).
4. Schulman, J., Jr., *J. Biol. Chem.*, **186**, 717 (1950).
5. Maloof, F., and Soodak, M., *Endocrinology*, **61**, 555 (1957).
6. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, **177**, 751 (1949).
7. Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, **119**, 1 (1937).
8. Chaney, A. L., *Ind. Eng. Chem. Anal. Ed.*, **12**, 179 (1940).
9. Peterman, M. L., Mizen, N. A., and Hamilton, M. G., *Cancer Research*, **13**, 372 (1953).
10. Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C., *J. Biol. Chem.*, **217**, 111 (1955).
11. Dempsey, E. W., and Peterson, R. R., *Endocrinology*, **56**, 46 (1955).
12. Wood, H. W., *Nature*, **175**, 1084 (1955).
13. Der-Garabedian, M. A., and Fromageot, C., *Compt. rend.*, **216**, 216 (1943).
14. Der-Garabedian, M. A., *Compt. rend. soc. biol.*, **138**, 445 (1944).
15. Sorbo, B., *Biochim. et Biophys. Acta*, **21**, 393 (1956).
16. Baxter, C. F., Van Reen, R., and Pearson, P. B., *Federation Proc.*, **15**, 216 (1956).
17. Baxter, C. F., Van Reen, R., Pearson, P. B., and Rosenberg, C., *Biochim. et Biophys. Acta*, **27**, 584 (1956).
18. Baxter, C. F., and Van Reen, R., *Biochim. et Biophys. Acta*, **28**, 567 (1958).
19. Baxter, C. F., and Van Reen, R., *Biochim. et Biophys. Acta*, **28**, 573 (1958).
20. Volkmann, C. M., and Beerstecher, E., Jr., *J. Biol. Chem.*, **223**, 1021 (1956).
21. Tong, W., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **227**, 773 (1957).
22. Astwood, E. B., *J. Pharmacol. Exptl. Therap.*, **78**, 79 (1943).
23. Wyngaarden, J. B., Wright, B. M., and Ways, P., *Endocrinology*, **50**, 537 (1952).
24. Astwood, E. B., *Ann. N. Y. Acad. Sci.*, **50**, 419 (1949).
25. Wood, J. L., and Williams, E. F., Jr., *J. Biol. Chem.*, **177**, 59 (1949).
26. Stanbury, J. B., *J. Biol. Chem.*, **228**, 801 (1957).
27. Stanbury, J. B., and Morris, M. L., *J. Biol. Chem.*, **233**, 106 (1958).
28. Painter, H. A., and Zilva, S. S., *Biochem. J.*, **46**, 542 (1950).
29. Knox, W. E., and LeMay-Knox, M., *Biochem. J.*, **49**, 686 (1951).
30. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., LaDu, B. N., Mitoma, C., and Udenfriend, S., *Science*, **121**, 603 (1955).

A Bacterial Pterin Deaminase*

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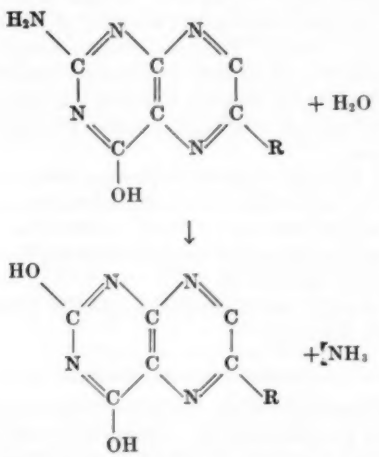
Much has been learned in the past several years of the function of complex pteridine-containing cofactors in the metabolism of single carbon units. However, few reactions are presently known which involve enzymatic alteration of the pterin ring system itself. Our approach to a study of this problem has been first to search, by enrichment culture techniques, for bacterial strains which metabolize pteridines, and then to investigate the detailed metabolic pathway with enzymes obtained from these bacteria. In this paper we report the isolation of an organism, *Alcaligenes metalcaligenes*, which can utilize 2-amino-4-hydroxypteridine-6-carboxylic acid as a source of carbon and nitrogen. Extracts of the bacteria contain an enzyme that catalyzes the hydrolytic deamination of many of the naturally occurring 2 - amino - 4 - hydroxypteridines (pterins) yielding the corresponding 2,4-dihydroxy compounds (lumazines), as illustrated by the following generalized reaction:



(The chemical nature of R is discussed in the text.)

A spectrophotometric method of assay of the reaction is described, and a procedure for the partial purification of the enzyme is reported. Several of the properties of the enzyme, including some indication of its substrate specificity, are also presented. The name pterin deaminase is proposed for this new enzyme.

* A preliminary account of this work has been presented (1).

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METHODS AND MATERIALS

Isolation and Cultivation of Microorganism—The organism[1] was originally obtained from a soil suspension enriched, under aerobic conditions, with pterin carboxylic acid[2] as the sole source of nitrogen and carbon. Extremely feeble growth was observed at 25° after an incubation period of 5 days. The pterin carboxylic acid-utilizing strain of microorganism was purified by several transfers of the culture to fresh, sterile media. Growth at this stage, however, was still exceedingly poor. A small quantity of cells was harvested by centrifugation and disrupted by grinding the pellet of bacteria with alumina. When this material was extracted with phosphate buffer (pH 6.3) and added to a quartz cuvette containing a solution of pterin carboxylic acid, distinct spectral alterations were observed between wave lengths 260 and 380 mμ (Fig. 1).

To study further the nature of this action, it was necessary to modify the culture conditions for the organism in order to obtain a sufficient yield of bacterial mass. The addition of glucose, broth and yeast extract to the medium greatly enhanced growth and resulted in cells, extracts of which still possessed comparable ability to attack pterin carboxylic acid. Further, by completely omitting the pteridine and supplementing the medium with inorganic ammonium salts, the growth of bacterial cultures remained excellent. Extracts of these cells still contained pterin deaminase in good quantity. The medium thereafter employed was prepared in the following manner. K_2HPO_4 (1.5 gm.), KH_2PO_4 (0.5 gm.), $MgSO_4 \cdot 7\ H_2O$ (0.2 gm.), NH_4NO_3 (2.0 gm.), Difco Nutrient Broth (5.0 gm.), and Difco Yeast extract (0.1 gm.) were dissolved in 500 ml. of tap water plus 450 ml. of glass-distilled water. Dextrose (0.2 gm. in 50 ml. of distilled water) was sterilized separately and subsequently added aseptically to the above autoclaved mixture just before the introduction of a few ml. of an inoculum of the organism.

Preparation of Cell-free Extracts—The bacteria were grown at 25° for approximately 55 hours with moderate aeration in 20-l. carboys each containing 10 l. of media. Cells were harvested with the aid of a Sharples Super centrifuge and washed several times with cold distilled water. The packed cells could be stored in this condition at −60° for at least 4 months without appreci-

[1] ATCC No. 13270. We are greatly indebted to Dr. Bruce Bellomy of the Clinical Pathology Department, National Institutes of Health, for his successful effort in the identification of the organism as *Alcaligenes metalcaligenes*.

[2] The abbreviations used are: pterin carboxylic acid, 2-amino-4-hydroxypteridine-6-carboxylic acid; lumazine carboxylic acid, 2,4-dihydroxypteridine-6-carboxylic acid; Tris, tris(hydroxymethyl)aminomethane; *p*-Cl-Hg-benzoate, *p*-chloromercuribenzoate.

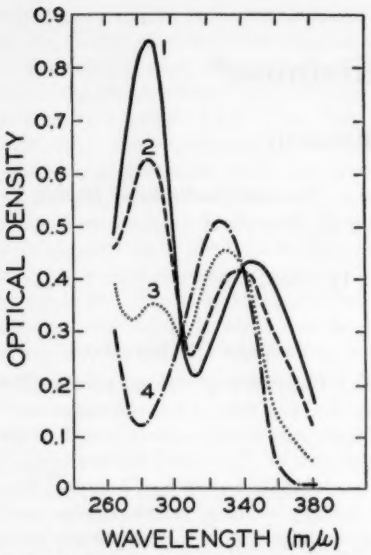


FIG. 1. Spectral alterations resulting from incubation of bacterial extract with pterin carboxylic acid. Cuvette contained 0.065 μmole of pterin carboxylic acid, 90 μmoles of K-phosphate buffer, pH 6.3, and 0.12 ml. of protamine-treated extract (step *1*) in a final volume of 1.0 ml. Temp. = 23°. Spectra were obtained at the start (*Curve 1*), and after 1, 2.5, and 7 hours of incubation (*Curves 2, 3, and 4*, respectively). Readings were taken with the Cary recording spectrophotometer, against a control cuvette in which the substrate was omitted.

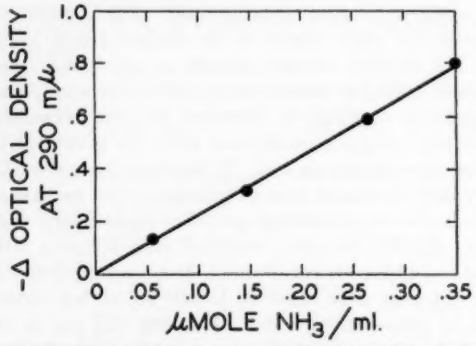


FIG. 2. Ammonia formation accompanying the decrease in absorption of pterin carboxylic acid at 290 mμ. Pterin carboxylic acid, 2.8 μmoles, K salt, were incubated with 560 μmoles of K-phosphate buffer, pH 6.3, and 1.5 ml. of a well dialyzed deaminase preparation from step *4*. Final volume, 7.5 ml. Absorbance measurements were obtained on 0.25-ml. aliquots diluted to 1.0 ml. with water. Points represent samples taken out at 2, 6, 12, and 22 hours, respectively, and assayed against control samples from a similar vessel incubated in the absence of pterin carboxylic acid.

able loss of pterin deaminase activity. Extracts were prepared by suspending 1 part, by weight, of cells in 1.8 parts of 0.05 M potassium phosphate buffer, pH 7.0, and disrupting the bacterial mass, in batches of 30 ml. for 25 minutes at 2°, in a Raytheon 10 KC sonic oscillator. The resulting material was centrifuged for 30 minutes at 2° in the Servall SS-1 centrifuge at 20,000 × *g*. The supernatant fluid was carefully decanted and submitted to the fractionation procedures described under "Experimental and Results."

Pteridine Compounds—Pterin carboxylic acid was prepared by the alkaline permanganate oxidation of pteroyl glutamic acid (Mann Chemical Company) as described by Weygand and Schaefer (2). To purify the resulting material as well as to obtain it in a form more water soluble than the free acid, the compound was first converted to its cyclohexylamine salt by addition of a slight excess of cyclohexylamine (Eastman Chemical Company) to a solution of the crude acid at pH 10. The clear mixture was chilled in ice, and several hours later a crop of white crystals of the salt was filtered and washed briefly with cold water. The free acid was then regenerated by acidification of a solution of this salt. After chilling to 0°, the white, flocculent precipitate was collected by centrifugation and once again converted to the cyclohexylamine salt by the same procedure as above. The crystalline product was washed thoroughly with small portions of cold water and cold acetone and then dried in a vacuum. When required, the salt was freed of cyclohexylamine by dissolving 200-mg. quantities of it in 5 ml. of water and passing this solution through a column (0.8 × 6.0 cm.) of Dowex 50 resin in the potassium form. The initial eluate usually contained over 75 per cent of the pterin carboxylic acid (determined spectrophotometrically) and gave no reaction for cyclohexylamine or ammonia with Nessler's reagent. This solution of the potassium salt of pterin carboxylic acid was freshly prepared every few weeks and stored at 0°. It was used in most of the experiments involving this pteridine reported below.

Pteroyl glycine and pteropterin (pteroyl triglutamic acid) were gifts of Dr. G. M. Briggs and Dr. Barbara Wright, respectively. All of the other pteridines used in this study were generously supplied from the Lederle Division of the American Cyanamid Company, through the courtesy of Drs. H. P. Broquist and R. B. Angier. For assay, about 2.5 mg. of pteridine were dissolved in 5 ml. of 0.05 M potassium phosphate buffer, pH 6.3. Those which were not sufficiently soluble at this pH were dissolved in 5 ml. of 0.05 M Tris buffer, pH 9.0, with warming if necessary.

Analytical Methods—Ammonia liberation from the pteridines was assayed by the Conway micro-diffusion technique (3), measuring the trapped ammonia by direct nesslerization.

Protein was determined spectrophotometrically by the method of Warburg and Christian (4).

Guanase activity was determined essentially by the method of Roush and Norris (5).

EXPERIMENTAL AND RESULTS

Nature of Reaction[3]—When protamine-treated extracts of *A. metalcaligenes* were incubated in a cuvette for several hours with pterin carboxylic acid and 0.1 M phosphate or Tris buffer, pH 6.3, a distinct and graded pattern of spectral changes was observed (Fig. 1). These shifts in absorption were characterized by three major spectral alterations: (*a*) a large decrease in extinction between 270 and 300 mμ, (*b*) a moderate decrease from 345 to 380 mμ, and (*c*) a relatively smaller increase between 310 and 340 mμ. No change in the spectrum of pterin carboxylic acid was noted when the bacterial extract was omitted from the

[3] Initial investigations upon pterin deaminase were carried out employing pterin carboxylic acid as substrate. Subsequently, many of the observations described in this section were repeated with 2-amino-4-hydroxy-6-methyl pteridine and pteroyl glutamic acid. Results with the latter substrates were complementary in all respects to those obtained with pterin carboxylic acid, but for the sake of brevity only the details of the experiments with pterin carboxylic acid are presented.

cuvette or when a heated extract (held at 100° for 3 minutes) was added in place of the nonheated enzyme preparation.

When viewed under a lamp emitting ultraviolet light, reaction mixtures containing enzyme, pterin carboxylic acid, and buffer exhibited a bright blue fluorescence at the start of incubation, which was replaced by a yellow-green fluorescence as the reaction proceeded to completion. Preliminary paper chromatographic studies (solvent: 3 per cent NH_4Cl) of the ultraviolet-fluorescing components in deproteinized incubation mixtures showed that this yellow-green fluorescence was due to a single substance which comprised by far the majority of the total fluorescing material present. Chromatography of a synthetic sample of lumazine carboxylic acid revealed that this lumazine derivative possessed the same fluorescence characteristics and mobility in this solvent system as did the product of the enzymatic reaction.

Ammonia was detected as an additional product of this reaction. With more purified enzyme preparations, ammonia formation was observed to proceed at a rate directly proportional to that of the change in absorption of the pterin substrate (Fig. 2), thus suggesting that these two processes were indeed interrelated. Isolation and chemical characterization of the deaminated pterin product, described below, provided direct evidence of the nature of the enzymatic reaction.

Isolation and Identification of Reaction Products—2,4-Dihydroxypteridine-6-carboxylic acid: Freshly-prepared extract, 27 ml., was added to 120 ml. of 0.01 M sodium phosphate buffer, pH 7.0, containing 125 mg. of the cyclohexylamine salt of pterin carboxylic acid. The mixture was layered over with 10 ml. of toluene and incubated at 23°. Samples were removed at several intervals during the following 48 hours to determine the extent of the reaction. This was done by measuring the decrement in light absorption at 290 mμ *versus* a control sample containing only extract and buffer. After the reaction had reached completion (48 hours), the toluene was removed and the incubation mixture acidified with 15 ml. of 20 per cent trichloroacetic acid. Protein was removed by centrifugation and the supernatant solution passed through a column of Dowex 50 ammonium form resin (1.0 × 9.5 cm.). The main portion of the ultraviolet-absorbing material remained upon the resin and was subsequently eluted by passage of 70 ml. of water through the column. This latter fraction was then treated with 3 ml. of a 20 per cent suspension of Nuchar at 35° for 5 minutes. The charcoal was sedimented by centrifugation, washed seven times with a total of 50 ml. of water, and finally eluted with 8 ml. of ethanol:water:ammonium hydroxide (concentrated), 40:20:5, at room temperature. The charcoal was removed by filtration. After chilling the filtrate in ice for several hours, the flocculent, pale yellow precipitate which formed was centrifuged and dried in a vacuum. The resulting 25 mg. of crude product were dissolved in 22 ml. of water, the solution clarified by centrifugation, and the acid precipitated by the addition of 4 ml. of 0.1 N HCl. The suspension was chilled for several hours and filtered. The precipitate was washed with cold acetone and dried in a vacuum. The yield of white, microcrystalline solid equaled 19 mg.

Analysis[4]

$C_7H_4O_4N_4 \cdot H_2O$

Calculated: C 37.2, H 2.67, N 24.7
Found: C 36.9, H 2.83, N 24.0

[4] Analyses were carried out by Dr. W. C. Alford of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

TABLE I

Characterization of pterin carboxylic acid product

Compound	Fluorescence	R_F		
		3% NH_4Cl	0.5 M phosphate buffer, pH 5.8	30% Ethanol-2% acetic acid
Pterin carboxylic acid	Blue	0.50	0.55	0.43
Lumazine carboxylic acid	Yellow-green	0.65	0.67	0.62
Pterin carboxylic acid product	Yellow-green	0.65	0.67	0.60

The absorption spectra of the isolated product in 0.1 M phosphate buffer, pH 6.3, and in 0.1 M NH_4OH were identical with those of synthetic lumazine carboxylic acid at all wave lengths between 220 and 380 mμ. The isolated compound also possessed the same R_F values and fluorescence on paper chromatograms as the authentic sample of the lumazine (Table I). No fluorescent or ultraviolet-absorbing impurities were detectable on these chromatograms.

2-Hydroxy-(2-deamino)-pteroyl glutamic acid: With pteroyl glutamic acid as the substrate, an extract of *A. metalcaligenes* catalyzed the formation of a new pterin. The product was obtained from an incubation mixture consisting of 177 mg. of pteroyl glutamic acid and 25 ml. of a freshly prepared sonic extract in 120 ml. of 0.01 M sodium phosphate buffer, pH 7.0. After an incubation of 48 hours at 23°, protein was precipitated by the addition of 17.5 ml. of 20 per cent trichloroacetic acid, and the pteridine product was isolated by much the same procedure as described for lumazine carboxylic acid in the preceding section.

Analysis[4]

$C_{19}H_{18}O_7N_6$

Calculated: C 51.6, H 4.10, N 19.0
Found: C 51.1, H 4.03, N 18.9

The absorption spectra of the isolated compound in 0.1 M phosphate buffer, pH 6.3, and in 0.1 M NH_4OH were identical with those of synthetic 2-hydroxy-(2-deamino)-pteroyl glutamic acid at all wave lengths between 220 and 380 mμ. On paper chromatograms (solvent: 3 per cent NH_4Cl), the enzymatic product migrated with an R_F of 0.54, identical with that of the authentic sample of the lumazine. No impurity absorbing or fluorescing under ultraviolet light could be detected on these chromatograms.

Quantitation of Spectral Data—The identification of the corresponding 2,4-dihydroxypteridines as products of the action of the bacterial extract upon pterin carboxylic acid and pteroyl glutamic acid furnished evidence that the nature of the reaction was that of an enzymatic deamination occurring at carbon 2 of the pterin substrates. To obtain quantitative information from the various spectral shifts noted during the course of action of the deaminase, complete ultraviolet-absorption spectra were determined in 0.1 M potassium phosphate buffer, pH 6.3, with solutions of pterin carboxylic acid and lumazine carboxylic acid of known concentration. These data are presented in graphic form in Fig. 3. Included are the values of the change (ΔE) in the molar extinction coefficient calculated for the wave lengths where the maximal difference spectrum is observed (*i.e.* at ap-

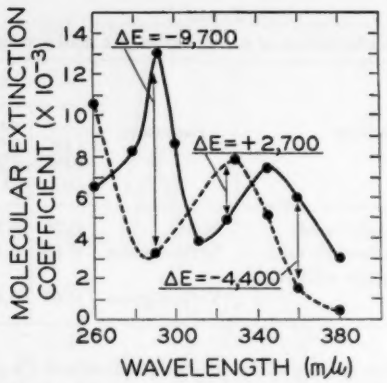


FIG. 3. Absorption spectra of pterin carboxylic acid and lumazine carboxylic acid at pH 6.3 as a basis for the spectrophotometric assay of pterin deaminase. ——, spectrum of pterin carboxylic acid, - - -, spectrum of lumazine carboxylic acid.

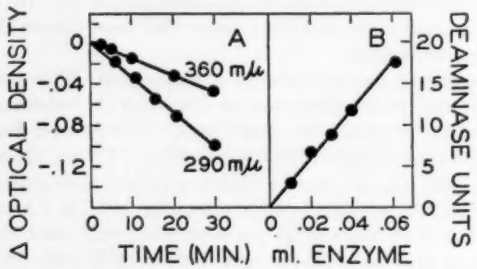


FIG. 4*A*. Proportionality of deaminase activity to time. 4*B*. Reaction rate as a function of enzyme concentration. Conditions as described in text.

TABLE II

Purification of pterin deaminase: removal of guanase

Step*	Yield	Specific activity†		B/A
		Pterin deaminase (A)	Guanase (B)	
Sonic extract	(100)	1.0	210	210
1. Protamine sulfate	82	1.3	290	220
2. Heat, I	75	3.0	100	33
3. Nuchar	70	5.1	180	35
4A. Alkaline ammonium sulfate	45	13	230	18
4B. Storage of 4A‡		13	8	0.6
5. Alumina gel Cγ	27	21	250	12
6. Heat, II	22	20	40	2

* Procedures described under "Experimental and Results."

† Units per mg. protein.

‡ Three weeks at 0° in 0.1 M Tris buffer, pH 9.0.

proximately 290, 325, and 360 mμ). These values thus represent the increment (or decrement) in optical density which would be observed, at the given wave length, for the hydrolytic deamination of 1 mmole of pterin carboxylic acid in a volume of 1.0 ml. at pH 6.3.

Assay of Pterin Deaminase—A spectrophotometric assay of pterin deaminase activity was devised, based upon the shifts in the spectrum of pterin carboxylic acid accompanying the conversion of the 2-amino group to the 2-hydroxyl group at pH values ranging about neutrality. The determination is performed at 23° in a 1-cc. Beckman quartz cuvette with a light path of 1.00 cm. Typical assay conditions are as follows:

Pterin carboxylic acid (2.0 μmoles per ml.), 0.05 ml.

0.1 M potassium phosphate buffer, pH 6.3, 0.80 ml.

Pterin deaminase preparation, 0.02 to 0.15 ml.

Water, up to a final volume of 1.0 ml.

Reaction is begun by addition of enzyme, and readings of optical density are taken every 3 minutes thereafter. (The control cuvette contains buffer and enzyme, but no pterin carboxylic acid.) With crude preparations, light measurement at 360 mμ is employed. However, with more purified fractions of the deaminase, it is possible to follow the course of the reaction at 290 mμ (the wave length at which there is observed the largest ΔE value between substrate and product (Fig. 3)). Linearity with time is observed for 30 minutes or longer, (Fig. 4*A*) depending upon the rapidity with which pterin carboxylic acid is deaminated. Linearity with respect to enzyme concentration is illustrated in Fig. 4*B*. A unit of activity is defined as that quantity of enzyme which, under these conditions, will catalyze the deamination of 1 mμmole of pterin carboxylic acid in 10 minutes.

Purification of Pterin Deaminase—In Table II are presented the results of a partial purification of pterin deaminase activity from extracts of *A. metalcaligenes*. An over-all purification of 20-fold was achieved, with a yield of 22 per cent. All steps were performed at 0–2° unless otherwise indicated.

Step 1: Treatment of extract with protamine sulfate—127 ml. of extract (8900 units, specific activity = 1.0) were treated, dropwise, with 60 ml. of a 1 per cent solution of protamine sulfate (Eli Lilly and Company) in 0.05 M potassium phosphate buffer, pH 7.0, with stirring over a period of 10 minutes. The suspension was stirred for an additional 10 minutes and then centrifuged for 20 minutes at 20,000 × *g*. The supernatant solution (170 ml.) contained the major portion of the deaminase (7300 units, specific activity = 1.3).

Step 2: First heat inactivation step—The enzyme solution from Step 1 was placed in a 750-ml. Erlenmeyer flask, rapidly warmed to room temperature, and treated with 34 ml. of 1.3 M potassium chloride. The flask and contents were immersed in a water bath maintained at 60°, and held at this temperature with gentle agitation of the solution for 7 minutes. The mixture was chilled in ice and denatured protein removed by centrifugation. The resulting pale yellow supernatant solution (170 ml.) contained 6670 units of enzyme with a specific activity of 3.0.

Step 3: Treatment with activated charcoal—The preparation from Step 2 was warmed to 27° and treated with 19 ml. of a 20 per cent suspension of Nuchar for 8 minutes, with gentle stirring. The mixture was then chilled in ice and clarified by centrifugation at 20,000 × *g* for 20 minutes. The major portion of the pterin deaminase was found in the resulting supernatant solution (175 ml., 6230 units, specific activity = 5.1).

Step 4: Alkaline ammonium sulfate fractionation—The pH of the above solution was adjusted to 8.4 by the careful addition of a few ml. of 1 N NH_4OH. Solid ammonium sulfate, 28 gm., was then added to each 100 ml. of this solution. Addition of the salt required 15 minutes, after which the suspension was equilibrated for another 10 minutes and centrifuged at 15,000 × *g*. The inactive precipitate (0 to 40 per cent fraction) was discarded and the supernatant solution treated, as described above,

with an additional 19 gm. of ammonium sulfate per 100 ml. of original volume. If further purification was not sought, the paste obtained by centrifugation of this fraction was dissolved in an amount of 0.1 M Tris buffer, pH 9.0, equal to about one-fifth the volume of the original solution of Step 3. After aging in ice for an hour, this 40 to 67 per cent fraction was freed from a small amount of inactive material by centrifugation. The resulting clear, very pale yellow supernatant solution (4000 units, specific activity = 13) could be stored at 0° for approximately 3 weeks with no appreciable loss of activity. For most of the enzymatic studies reported below, pterin deaminase preparations were employed which had been purified as far as this stage. Two further steps were included only when it was desirable to obtain a fraction as free as possible from residual guanase activity.

Step 5: Treatment with alumina gel Cγ—The ammonium sulfate 40 to 67 per cent paste from Step 4 was dissolved in 50 ml. of 0.005 M potassium phosphate buffer, pH 7.0, and stirred for 15 minutes with an equal volume of alumina gel Cγ suspension (16 mg. of gel per ml.). After centrifugation of this mixture, the supernatant was discarded and the gel washed twice with small volumes of cold water. Elution of the activity was accomplished by stirring the gel for 45 minutes with 30 ml. of 0.085 M phosphate buffer, pH 7.5. Upon centrifugation, the supernatant solution contained 2400 units of deaminase, with a specific activity of 21.

Step 6: Second heat inactivation step—Conditions for this final operation were much the same as those described under Step 2 with the exception that the pH of the gel eluate was adjusted to 9 with 1 M Tris buffer, pH 9.5, just before heating. The supernatant (32 ml.) obtained upon removal of inert, denatured protein contained 1960 units of pterin deaminase, specific activity = 20.

Properties of Pterin Deaminase

Stability and Activity at Various pH Values—During initial studies on purification of the enzyme, it was observed that severe losses of activity occurred upon storage of the protein solutions at pH 7 for a few days in an ice bath. Freezing of the protein solution at −20°, variations in ionic strength and type of buffer employed, or the presence of thioethanol or glutathione did not enhance enzyme stability at this pH. It was soon observed, however, that destruction of the activity on storage could be markedly diminished by increasing the pH of the solution. The most satisfactory conditions for storage of enzyme fractions were found to be at pH 9.0 in 0.1 M Tris buffer at 0°. Under such conditions pterin deaminase activity remained almost unchanged for several weeks.

The pH optimum for pterin deaminase activity occurs between pH 6.3 and 6.7. Although the reaction proceeds in the complete absence of phosphate, the rate of deamination is slightly faster in phosphate than in Tris buffer.

Nonidentity of Pterin Deaminase and Guanase—Extracts of *A. metalcaligenes* contain a highly active guanase (Table II, column 4). Because the chemical structure of the pterin ring system is so similar to that of guanine, the possibility was investigated that the pterin deaminase and guanase components reflected the activity of one and the same enzyme. Accordingly, the rates of deamination of guanine and pterin carboxylic acid, respectively, were compared in the various fractions obtained during the course of purification of pterin deaminase. It can be seen from the data shown in the final column of Table II that the ratio of the specific activity of guanase to that of pterin deaminase decreased markedly throughout the fractionation procedures. The most striking differences occurred in the stability to heat (Steps 2 and 6) and to storage or handling at alkaline pH (Step 4*B*).

Additional evidence indicating the nonidentity of the two deaminase activities was obtained from studies of the effect of guanine and pterin carboxylic acid on the rate of deamination of another pterin substrate, 2-amino-4-hydroxy-6-methyl pteridine. At concentrations several times that of the substrate, pterin carboxylic acid exerted a definite inhibition, whereas guanine had no effect on the rate of this reaction. These data, together with those on fluoride sensitivity, referred to below, have permitted the conclusion that the pterin deaminase and guanase activities of this strain of Alcaligenes are probably distinct enzymatic entities.

Inhibitors—Pterin deaminase activity was not significantly affected by extensive dialysis of enzyme fractions against 0.1 M Tris buffer, pH 9.0. Preincubation with Versene (ethylenediaminetetraacetate), KCN, or 8-hydroxyquinoline sulfate likewise did not result in a decrease of activity. However, *p*-Cl-Hg-benzoate and sodium or potassium fluoride each caused severe inhibition when either preincubated with the deaminase or added directly to the experimental cuvette containing pterin carboxylic acid and enzyme. The inhibitory action of *p*-Cl-Hg-benzoate could be effectively prevented with glutathione. The fluoride inhibition could not be overcome by extended incubation or the addition of a large excess of Mg^{++}, but was completely removed upon dialysis of a fluoride-treated enzyme preparation against two changes of a large volume of 0.01 M Tris buffer, pH 9.0. A 50 per cent inhibition of the initial rate of pterin carboxylic acid deamination was observed at fluoride ion concentrations of approximately 3×10^{-5} M. This is in contrast to an inhibitor concentration of over 10^{-3} M required to effect a similar reduction in the guanase activity of these preparations. None of the other halide ions showed significant inhibitory properties.

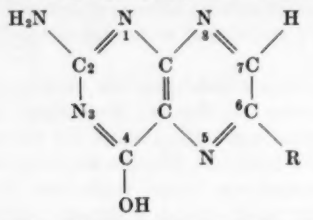
Irreversibility of Deamination—Evidence for a reversible deamination of pterins catalyzed by this bacterial enzyme could not be obtained by the use of the sensitive spectrophotometric method. Extended incubation of the purified enzyme with lumazine carboxylic acid and varying concentrations of ammonium ion led to no detectable shifts in the absorption of the lumazine at 290, 325, and 360 mμ, wave lengths at which appreciable changes would be observed (see Fig. 3) if reamination to the corresponding pterin had occurred.

Specificity of Pterin Deaminase—Some of the structural features required for a pteridine compound to serve as a substrate were investigated by testing a limited number of available derivatives (Table III). The ability of the test compound to undergo enzymatic deamination was generally determined in one of two ways: (*a*) by direct measurement of liberated ammonia, and (*b*) spectrophotometrically, by noting the appearance, with time, of certain characteristic changes between 260 and 380 mμ, similar to those shown in Fig. 1 for pterin carboxylic acid. In cases where both of these criteria were employed, perfect correlation between the two methods was obtained. Certain structural requirements for a given pteridine to function as a substrate for the deaminase can be deduced from the information presented in this table:

1. Only those pteridines possessing the pterin structure (*i.e.* the 2-amino and 4-hydroxyl functional groups) are deaminated.

TABLE III

Substrate specificity of pterin deaminase



Pteridines attacked	Pteridines not attacked
2-Amino-4-hydroxy-6- -H	(a) 2-amino-
2-Amino-4-hydroxy-6- $-CH_3$	(b) 2-amino-4-hydroxy-7-methyl-
2-Amino-4-hydroxy-6- $-CH_2OH$	(c) 2-amino-4-hydroxy-7-carboxy-
2-Amino-4-hydroxy-6- -CHO	(d) 2-amino-4-hydroxy-6,7-dimethyl-
2-Amino-4-hydroxy-6- -COOH	(e) 2-hydroxy-4-amino-6-methyl-
2-Amino-4-hydroxy-6- -CH(OH)-CH(OH)-CH_2OH	(f) 2,4-diamino-
2-Amino-4-hydroxy-6- $-CH_2COOH$	(g) aminopterin
Pteroic acid	(h) amethopterin
Pteroyl-(glutamic)$_{1,2,3}$	(i) xanthopterin
Pteroyl-aspartic	(j) leucovorin
Pteroyl-glycine	

Juxtaposition of these groups (compound e), removal of the 4-hydroxyl (compound a) or its substitution by an amino group (compounds f, g, h) produce pteridines not attacked by the enzyme.

2. The nature of the substitution at carbon 6 is relatively unimportant. However, an *hydroxyl* group at this position (compound i) results in an inactive substance.

3. Carbon 7 must be unsubstituted, since blocking of this position by a methyl (compounds b and d) or carboxyl function (compound c) destroys the ability of the pterin to serve as a substrate.

4. An *N*-5-formylated and reduced pterin (compound j) is not deaminated.

DISCUSSION

The existence of hydrolytic enzymes irreversibly deaminating amino-purines and amino-pyrimidines has been well documented (5–8). Although the observation has been made that certain pterins inhibit the deamination of guanine by crude preparations of rat liver guanase (9), no evidence has been reported, until now, that pterin derivatives undergo enzymatic deamination to lumazines. The general characteristics of the enzymic reaction described in this paper, including its apparent irreversibility and the sensitivity of the enzyme to fluoride, are sufficiently similar to those in the purine and pyrimidine series to permit its inclusion in the same category as the latter deamination processes (10).

The data indicate that the activity of pterin deaminase is associated with a protein fraction distinct from that which catalyzes the deamination of guanine. The evidence for this conclusion rests on the following three criteria: (*a*) a marked decrease in the ratio of guanase to pterin deaminase activity upon purification of the latter, (*b*) difference in the relative sensitivity of the two activities to fluoride ion, and (*c*) inhibition by pterin carboxylic acid, but not by guanine, of the rate of deamination of 2-amino-4-hydroxy-6-methyl pteridine. Whether the reverse situation can be true, *i.e.* that guanase may possess a substrate specificity sufficiently broad to attack pterins, is not known with certainty at this time. Rat liver, *Escherichia coli*, and *Torula utilus* extracts containing guanase were incapable of deaminating pterin carboxylic acid.[5] However, similar preparations of other strains of Alcaligenes possessed deaminase activity for both the purine and pteridine compounds, and, in certain of the latter cases, separation of the enzymes could not be achieved by a variety of purification procedures. It would thus appear that the question of guanase action on pterins is dependent upon the organism, species, and strain under consideration.

The role of pterin deaminase within the bacterial cell cannot be ascertained at this time. Since a variety of pteroyl glutamic acid derivatives are attacked by the enzyme, leading to the formation of the corresponding microbiologically inactive lumazine analogs (11), it is conceivable that the deaminase may function in a regulatory manner to help in maintaining proper intracellular levels of pteridine-containing cofactors. The irreversibility of the reaction argues against the possibility of the direct involvement of this enzyme in the biosynthesis of pterins from certain recently reported lumazine compounds of natural occurrence (12–14).

SUMMARY

An enzyme catalyzing the deamination of pterins has been partially purified from extracts of a strain of Alcaligenes. The reaction is of an irreversible, hydrolytic nature, similar in many respects to those catalyzed by certain amino-purine and amino-pyrimidine deaminases.

A spectrophotometric method has been developed for the assay of pterin deaminase, based upon the shifts that occur in the ultraviolet when the enzyme attacks 2-amino-4-hydroxy-pteridine-6-carboxylic acid at neutral pH.

Several properties of the enzyme, including its marked sensitivity to fluoride ion, have been described. Evidence has been presented which suggests that the enzyme can be regarded as distinct from a highly active guanase also found in extracts of the organism. Certain structural features of the pteridine molecule requisite for its activity with pterin deaminase have been noted.

REFERENCES

1. LEVENBERG, B., *Federation Proc.*, **17**, 263 (1958).
2. WEYGAND, F., AND SCHAEFER, G., *Ber. deut. chem. Ges.*, **85**, 307 (1952).
3. CONWAY, E. J., *Microdiffusion analysis and volumetric error*, 3rd edition, D. Van Nostrand Company, London, 1950.
4. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **310**, 384 (1941–1942).
5. ROUSH, A. H., AND NORRIS, E. R., *Arch. Biochem.*, **29**, 124 (1950).
6. SCHMIDT, G., in E. CHARGAFF AND J. N. DAVIDSON (Editors), *The nucleic acids, Vol. I*, Academic Press, Inc., New York, 1955, p. 555.
7. KREAM, J., AND CHARGAFF, E., *J. Am. Chem. Soc.*, **74**, 5157 (1952).

[5] Unpublished experiments of the authors.

8. Heppel, L. A., Hurwitz, J., and Horecker, B. L., *J. Am. Chem. Soc.*, **79**, 630 (1957).
9. Dietrich, L. S., and Shapiro, D. M., *J. Biol. Chem.*, **203**, 89 (1953).
10. Roush, A. H., *Abstracts of papers of the 133rd meeting of the American Chemical Society*, American Chemical Society, Washington, 1958, p. 9C.
11. Angier, R. B., Boothe, J. H., Mowat, J. H., Waller, C. W., and Semb, J., *J. Am. Chem. Soc.*, **74**, 408 (1952).
12. Masuda, T., *Pharm. Bull. Tokyo*, **4**, 71, 375 (1956).
13. Forrest, H. S., and McNutt, W. S., *J. Am. Chem. Soc.*, **80**, 739 (1958).
14. Maley, G. F., and Plaut, G. W. E., *Federation Proc.*, **17**, 268 (1958).

The Respiratory Chain in Phosphorylating Subfragments of Mitochondria Prepared with Digitonin*

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Previous communications from this laboratory (1–6) have described various enzymatic reactions associated with oxidative phosphorylation in subfragments of rat liver mitochondria obtained by treatment with digitonin. This paper describes the electron transport system of such mitochondrial fragments.

Although some chemical and physical treatments affecting the morphology of mitochondria have been observed to cause substantial changes in the molar ratios of the various respiratory carriers (7–9), the results reported in this paper show that mitochondrial subfragments obtained by treatment of rat liver mitochondria with digitonin contain complete and substantially intact respiratory chains. With the exception of pyridine nucleotide, the relative molar concentrations of the carriers in the fragments, in comparison with those in intact mitochondria, is not significantly altered by the digitonin treatment. The occurrence of a pyridine nucleotide transhydrogenase in the subfragments is also demonstrated.

EXPERIMENTAL

Methods—The mitochondrial subfragments were prepared and assayed for oxidative phosphorylation as described previously (10, 1) and only those preparations manifesting maximal phosphorylating efficiency were employed. Difference spectra were measured on the expanded per cent transmission scales of the Beckman DK-2 ratio recording spectrophotometer and were replotted in terms of optical density. Due to the turbidity of the preparations, control experiments were performed to eliminate errors due to light scattering and the effects of stray light (11). Changes in light scattering caused by dilution during the course of the spectrophotometric experiments were kept to a minimum by adding reagents in relatively small volumes (from 1 to 5 μl.). To test for possible shifts in the position of the absorption maxima or minima due to light scattering, the spectrum of a dilute solution of Fe^{+++} cytochrome *c* was recorded between 380 and 630 mμ with the mitochondrial subfragments present in both the reference and sample cuvettes. There were no significant differences between this spectrum and one of oxidized cytochrome *c* recorded in the absence of the fragment preparation. Thus the degree of turbidity of these preparations is not sufficient to cause significant shifts in absorption maxima.

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The rate of reduction of Fe^{+++} cytochrome *c* and pyridine nucleotides was followed spectrophotometrically as previously described (2). The concentration of cytochromes *a*, a_3, *b*, and *c*, and flavoprotein were determined spectrophotometrically, employing the wave length pairs and Δ extinction coefficients of the reduced minus the oxidized carriers given by Chance and Williams (7). The total iron content of the enzyme complex was determined by the *o*-phenanthroline method after wet oxidation (12).

Enzymatic Reduction of Respiratory Carriers—In order to measure the difference spectrum of those carriers functioning in the phosphorylating respiratory chain of the mitochondrial subfragments, the carriers were reduced with D(−)-β-hydroxybutyrate in an incubation medium which had been previously established as optimum for the measurement of oxidative phosphorylation (1). In Fig. 1, the reference cell contained a system composed of mitochondrial subfragments, ADP, and inorganic phosphate in the presence of oxygen. The carriers were assumed to be completely oxidized in the reference cell, since the enzyme preparations contain negligible endogenous substrate. Chance and Williams (13) found that the respiratory chain of intact mitochondria was nearly completely oxidized in the absence of added substrate when ADP is present. To the *sample* cuvette, which contained the same quantities of enzyme, ADP, and phosphate, β-hydroxybutyrate was added and the system was flushed briefly with tank nitrogen to remove a large part of the dissolved oxygen. *Curve A* of Fig. 1 is a difference spectrum with the use of the nonrespiring sample with its carriers in the oxidized state as base-line, against which was recorded the spectrum of the respiring system in the sample cuvette. This curve indicates the aerobic steady state oxidation-reduction levels of the cytochromes during oxidation of β-hydroxybutyrate (commented on below), which remain constant until all of the dissolved oxygen has been utilized, whereupon the carriers become fully reduced to produce the difference spectrum of *Curve B*.

Typical peaks and depressions were observed in the completely reduced spectrum of *Curve B* which correspond to the reduced forms of cytochromes *c*, *a*, and a_3, and flavoprotein (7). Upon the addition of cyanide to the anaerobic, fully reduced system, the only change in the spectrum was a decrease in the 445 mμ peak, due to the formation of the cyanide complex of reduced cytochrome a_3. Thus, under anaerobic conditions the presence of cytochromes *c*, *a*, and a_3, and of flavoprotein in the mitochondrial subfragments can readily be demonstrated, under conditions similar to those used by Chance and Williams (7) in their study of intact mitochondria.

This spectrum does not demonstrate unequivocally the pres-

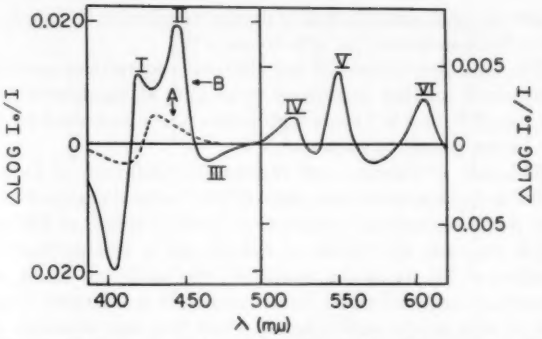

FIG. 1. Difference spectra of mitochondrial subfragments. The sample cuvette contained 0.01 M D(−)-β-hydroxybutyrate, 0.0024 M ADP, 0.01 M inorganic phosphate (pH 6.5) and mitochondrial subfragments (100 μg. N/ml.) in a total volume of 3.0 ml. The reference cuvette contained the same medium without D(−)-β-hydroxybutyrate, representing the oxidized complex. In *Curve A* (broken line), the difference spectrum is due to changes in the oxidation-reduction concentrations of the respiratory enzymes upon initiation of rapid respiration with D(−)-β-hydroxybutyrate, minus the nonrespiring reference sample containing the carriers in the fully oxidized state. This spectrum remains unchanged until all of the dissolved oxygen is utilized. *Curve B* is caused by the ensuing anaerobiosis of the sample cuvette which gives the difference between the spectrum of the enzymatically fully reduced carriers and the spectrum of the oxidized carriers. The roman numerals indicate the peaks corresponding to the reduced forms of carriers as follows. I, γ-peak of ferrocytochrome *c*; II, γ-peak of reduced cytochrome a_3 and *a*; III, reduced flavoprotein; IV, β-peak of reduced cytochromes *b* and *c*; V, α-peak of ferrocytochrome *c*; VI, α-peak of reduced cytochromes *a* and a_3.

ence or absence of cytochrome c_1 in the digitonin preparation, since the absorption bands of reduced cytochrome c_1 would be obscured by and fuse into those of cytochrome *c* (*cf.* 7, 14, 15). The possibility therefore exists that cytochrome c_1 contributes to the absorption peak having a maximum at 550 mμ, but more refined methods (14–16) would be required to establish its presence with certainty.

No distinct peaks for reduced cytochrome *b* were observed in the anaerobic spectrum; the 430 and 563 mμ peaks of reduced cytochrome *b* are presumably obscured by the γ peaks of reduced cytochromes *c*, *a*, and a_3 and the α peak of reduced cytochrome *c*, respectively. In intact mitochondria, Chance and Williams (7) were unable to observe peaks for reduced cytochrome *b* in a difference spectrum of the fully reduced (state 5) minus the aerobic steady-state (state 3) (in which cytochrome *b* was reduced only 16 per cent), for similar reasons. However, as is shown below, use of the respiratory inhibitor antimycin A readily permitted the visualization of the bands of reduced cytochrome *b* in the digitonin fragments, as was the case in intact mitochondria (7).

Aerobic Steady-state and Effect of Antimycin A—The aerobic steady-state condition of the respiratory pigments is again reproduced in *Curve A* of Fig. 2. A higher enzyme concentration was used in this experiment. The addition of 0.18 μg. of antimycin A per ml. to the sample cuvette produced the spectrum shown as *Curve B* in Fig. 2. Antimycin A inhibits the mitochondrial electron transport system at the level of cytochrome *b* (7) and has been shown to inhibit completely the oxidation of β-hydroxybutyrate by the digitonin particle (1, 10). With antimycin A to block reoxidation of cytochrome *b*, the α-, β-, and γ-peaks of reduced cytochrome *b* at 563, 530 and 430 mμ, respectively, and the depression at 460 to 465 mμ caused by reduced flavoprotein are now quite clearly observed, with no interference from the reduced bands of cytochromes *c*, *a*, and a_3 which are all fully oxidized in this system. Thus the respiratory chain of the digitonin subfragments contains functional cytochrome *b*, as well as cytochromes *c*, *a*, and a_3.

A similarity can be observed between the difference spectrum of the respiratory enzymes in the aerobic steady state and that of the antimycin A inhibited system, which indicates that cytochrome *b* is apparently the only measurably reduced cytochrome in the aerobic steady state condition. With the $\Delta OD_{430-490\ m\mu}$ (13) as a measure of reduced cytochrome *b*, and since it may be assumed that cytochrome *b* is completely oxidized in the reference cell (13), it was determined from a number of such experiments with the digitonin preparation that cytochrome *b* is approximately 45 per cent reduced in this aerobic steady state. For comparison, Chance and Williams (13) have found that cytochrome *b* is reduced about 16 per cent in intact rat liver mitochondria under similar conditions, *i.e.*, "state 3" (high concentration of ADP and substrate) minus "state 2" (high ADP, no substrate).

Concentration and Molar Ratios of Respiratory Carriers—In Table I is a summary of the average concentration of the respiratory carriers in the electron transport system of four different digitonin preparations. There was very little variation between preparations. Cytochromes a_3, *a*, and *c* (c_1) were measured in the totally reduced system as in Fig. 1; cytochrome *b* and flavoprotein were determined in the antimycin A inhibited system, which permits the determination of these two carriers without interference from the other cytochromes. As can be seen from a comparison of the last two columns, the molar ratios of the cytochromes and flavoprotein in the digitonin preparation closely

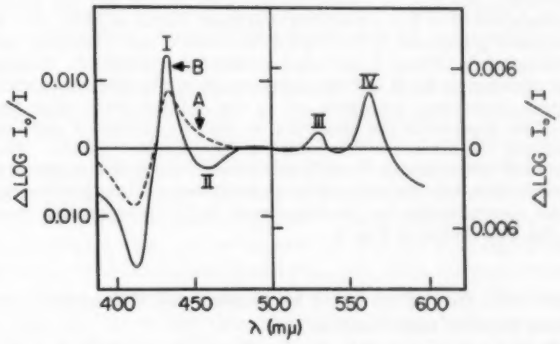

FIG. 2. Difference spectra of the aerobic steady state and the effect of antimycin A. Sample cuvette contained 0.01 M D(−)-β-hydroxybutyrate, 0.0024 M ADP, 0.01 M inorganic phosphate (pH 6.5) and mitochondrial subfragments (220 μg. N/ml.) in a total volume of 3.0 ml. The reference sample contained the same medium without D(−)-β-hydroxybutyrate, representing the oxidized complex. *Curve A* (broken line) represents the partially reduced respiratory enzymes in the aerobic steady state during D(−)-β-hydroxybutyrate oxidation, as in Fig. 1. *Curve B* (solid line) is caused by the addition of antimycin A (0.18 μg./ml.) to the sample cuvette, which completely inhibits respiration. The roman numerals indicate the peaks or depressions corresponding to the reduced forms of the carriers as follows: I, γ-peak of reduced cytochrome *b*; II, reduced flavoprotein; III, β-peak of reduced cytochrome *b*; IV, α-peak of reduced cytochrome *b*. Note that the enzyme concentration in this experiment is 2.2 times greater than in the experiment of Fig. 1.

TABLE I

Concentration of components of respiratory chain

The concentrations were determined as described in "Methods," and are the average for four different preparations. Molar ratios based on cytochrome $a = 1$.

Component	Mitochondrial subfragments		Normal liver mitochondria*
	mμmoles/mg. N	*molar ratios*	*molar ratios*
Cytochrome a_3	1.5	1.0	1.2
Cytochrome a	1.6	1.0	1.0
Cytochrome b	0.9	0.7	0.9
Cytochrome c (c_1)	2.8	1.8	1.7
Flavoprotein	4.5	3.0	3.6
DPN	0.45	0.3	19

* Values for normal liver mitochondria from Chance and Williams (7).

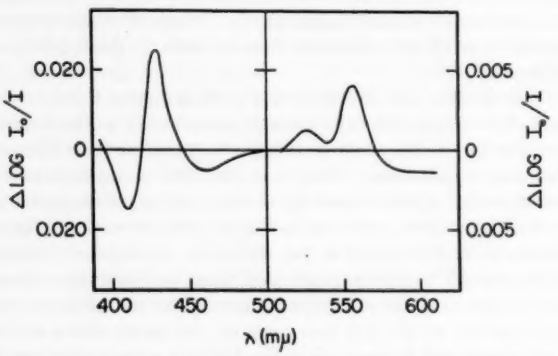

FIG. 3. Difference spectrum of the pigments reduced by hydrosulfite, but not by anaerobiosis. The sample and the blank contained 0.01 M D(−)-β-hydroxybutyrate, 0.0024 M ADP, 0.01 M inorganic phosphate (pH 6.5), 0.0005 M KCN and mitochondrial subfragments (210 μg. N/ml.), in a total volume of 3.0 ml. Due to the presence of KCN in both cuvettes, all of the carriers participating in electron transport are in the reduced state; thus the baseline represents the spectrum of the enzymatically reduced carriers. Hydrosulfite was added to the sample cuvette. The recorded difference spectrum is due to those pigments reduced by hydrosulfite, but not reduced by anaerobiosis. Note that the enzyme concentration in this experiment is 2.1 times greater than in the experiment of Fig. 1.

agree with the values found by Chance and Williams (7) for intact rat liver mitochondria.

Numerous unsuccessful attempts have been made to measure spectrophotometrically the bound DPN of the digitonin preparation (17). No peak occurs at 340 mμ in the aerobic steady state or in anaerobiosis; increasing the concentration of the enzyme complex, with subsequent increase in turbidity of the sample, places 340 mμ beyond the useful range of the instrument, since at this wave length light scattering effects are severe. However, measurements of the bound DPN were carried out with a catalytic test employing diaphorase,[1] after extraction of DPN from the multienzyme complex, and revealed the presence of about 0.3 mole of DPN per mole of cytochrome *a*. The amount of bound DPN in the mitochondrial subfragments is thus very small in contrast to intact rat liver mitochondria, where the total concentration of pyridine nucleotide is frequently 30 to 40 times that of the cytochrome *a* (7).

The total iron content of four different preparations was also determined, and has been found to be 35 to 45 mμmoles of iron per mg. of N, or 5 to 7 times higher than can be accounted for by the known cytochromes present.

Reduction of Carriers with Hydrosulfite—Addition of hydrosulfite to systems which were already fully reduced enzymatically with β-hydroxybutyrate caused a new peak to appear at 430 mμ, which obscured the γ-peak of cytochrome *c*, and produced a shoulder at 556 mμ on the α-peak of cytochrome *c*. These observations suggested that a heme compound of unknown origin was present in the subfragments which was not reducible by β-hydroxybutyrate.

In Fig. 3 is shown the difference spectrum of the unidentified pigment, without interference from the functional cytochromes. In this experiment both the blank and sample cuvettes contained phosphate, ADP, β-hydroxybutyrate, and cyanide to reduce fully the enzymatically functional respiratory pigments. Hydrosulfite was now added to the sample cuvette, to give a difference spectrum of the unidentified pigment with peaks at 426, 530, and 556 mμ. As can be seen from Fig. 3, there were no peaks which could be attributed to a further reduction of cytochromes *c*, *a*, or a_3, indicating that these cytochromes are completely reduced by β-hydroxybutyrate.

Three possibilities have been eliminated as the source of this spectrum: (*a*) the peaks are not attributable to a further reduction of cytochrome *b*, which has a peak at 430 mμ and not 426 mμ; (*b*) the conversion of oxyhemoglobin to hemoglobin should occur on anaerobiosis, as in Fig. 1, and would not require addition of dithionite; (*c*) the difference spectrum of hemoglobin minus methemoglobin does not produce peaks with these maxima. Chance and Williams (7) have found a similar compound in intact mitochondria which is reduced by hydrosulfite and not by anaerobiosis, and conclude that it could be cytochrome b_5, mitochrome (18), or unknown hemeproteins. It is possible that the digitonin treatment of the mitochondria denatures a part of the cytochromes. Hülsmann *et al.* (19) have demonstrated that the component in mitochrome (18) responsible for its characteristic spectrum is derived from the cytochromes. The amount of this compound varies considerably among different digitonin preparations, and it can be concluded that it is probably not a member of the phosphorylating electron transport system in the preparation, but may be an adventitious, possibly denatured, hemeprotein.

The average concentration of the hydrosulfite reducible material, determined with the use of $\Delta E_{426\text{-}405\ m\mu} = 200\ mM^{-1}\ cm.^{-1}$ (7), was 2.6 mμmoles per mg. of N, which is about equal to the concentration of cytochrome *c* in the digitonin preparation. This material can thus account for only a small portion of the noncytochrome iron present in these preparations.

Pyridine Nucleotide Transhydrogenase Activity of Enzyme Complex—Although DPNH is rapidly oxidized by the digitonin preparation (1, 2), it has been found that TPNH is only very slowly oxidized. However, the addition of a catalytic quantity of DPN^+ appreciably accelerates the rate, suggesting the presence of pyridine nucleotide transhydrogenase, which Kaplan *et al.* (20) have shown to occur in rat liver mitochondria. Transhydrogenase activity of the mitochondrial subfragments was assayed in a system composed of the mitochondrial subfragments, Fe^{+++} cytochrome *c*, cyanide to inhibit reoxidation of reduced

[1] J. Stern, personal communication of unpublished method.

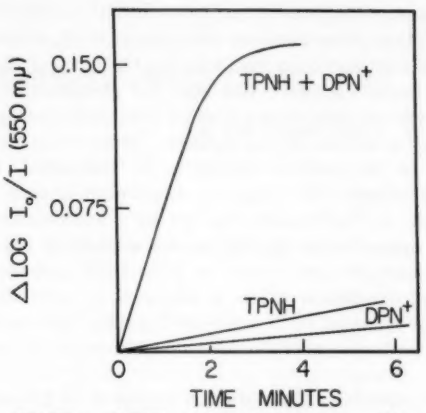

FIG. 4. TPNH to DPN+ transhydrogenase activity of mitochondrial subfragments. The reaction system contained 0.01 M inorganic phosphate (pH 6.5), 0.003 M ADP, 0.0001 M KCN, 9×10^{-6} M Fe^{+++} cytochrome *c* and mitochondrial subfragments (50 μg. total N). The reaction was initiated by the addition of 35 mμmoles of DPN+, 25 mμmoles of TPNH or a combination of both DPN+ and TPNH. Final volume 1.0 ml. The recording spectrophotometer was adjusted to give an optical density of zero at zero time. The curves are redrawn from an actual tracing.

Fe^{+++} cytochrome *c* by cytochrome *a* and a_3, phosphate, and ADP. To this system was added either DPN+, TPNH, or a combination of both nucleotides. It had previously been demonstrated that DPNH alone will rapidly reduce Fe^{+++} cytochrome *c* under these conditions (2). As is shown in Fig. 4, with the addition of DPN+ or TPNH alone there was only very slow reduction of Fe^{+++} cytochrome *c*, but upon addition of both nucleotides together a rapid reduction of Fe^{+++} cytochrome *c* occurred. In this system TPNH presumably reduced the DPN+, via transhydrogenase, and the reduced DPN+ in turn reduced Fe^{+++} cytochrome *c* via a DPN-cytochrome *c* reductase (2). Similar experiments showed that DPNH alone is oxidized at about 8 times the rate of oxidation of TPNH in the presence of DPN+. Therefore it can be assumed that the rate-limiting reaction in oxidation of TPNH is the pyridine nucleotide transhydrogenase.

With a test system containing the mitochondrial subfragments, cyanide, glucose 6-phosphate and glucose 6-phosphate dehydrogenase to reduce TPN+, and exactly known quantities of TPN+ and DPN+, the total optical density of the reduced pyridine nucleotide peak at 340 mμ was measured. The optical density observed could only be accounted for if both TPN+ and DPN+ were reduced. Since TPNH was continuously generated and DPN+ alone was not reduced in the system, the DPN+ was being reduced by the TPNH via the pyridine nucleotide transhydrogenase.

The particle-bound pyridine nucleotide transhydrogenase was found to be inhibited by L-thyroxine, confirming the report of Ball and Cooper (21). At 1×10^{-5} and 5×10^{-5} M L-thyroxine transhydrogenase activity was inhibited 45 and 85 per cent respectively. L-Thyroxine does not inhibit the DPN-cytochrome reductase present in the subfragments of mitochondria.

These results demonstrate that the mitochondrial subfragments contain a pyridine nucleotide transhydrogenase which catalyzes the transfer of electrons from TPNH to DPN+, in addition to the enzymatic systems for electron transport from DPNH to oxygen and coupled phosphorylation.

DISCUSSION

The results described indicate that the phosphorylating subfragments derived from mitochondrial membranes with digitonin contain a complete complement of those electron transport enzymes observed in intact mitochondria (7) and, with the exception of DPN, the carriers are in approximately the same relative molar proportions. This was a rather unexpected observation, for it has been observed that other treatments which disrupt the morphology of mitochondria alter the relative proportions of the carriers (7–9). It may be concluded that the digitonin treatment fragments the mitochondrial membranes along relatively fragile lines of attachment between organized assemblies of phosphorylating respiratory enzymes (10, 22), without disturbing greatly the composition of the individual respiratory assemblies.

In the digitonin subfragments the rate-limiting step of the respiratory chain appears to be between cytochrome *b* and *c*. However, the relatively high degree of reduction of the cytochrome *b* which exists during the aerobic steady state may not be solely a reflection of its participation in electron transport, since it is possible that adventitious cytochrome *b* (*i.e.* "dislocated" from the chain) could be reduced rapidly by the electron transport system and remain in this state during respiration without undergoing rapid reoxidation. Chance (23) has presented evidence for reduction of "dislocated" cytochrome *b* in nonphosphorylating heart muscle preparations. It is probable that at least part of the cytochrome *b* in the digitonin fragments is in such a "dislocated" state, since the flavoprotein in the subfragments was not as fully reduced during the aerobic steady state oxidation as was cytochrome *b*. However, since approximately 55 per cent of the total cytochrome *b* in the digitonin subfragments becomes reduced when antimycin A is added to the system in the aerobic steady state, it may be concluded that a substantial fraction of the cytochrome *b* in these preparations is directly involved in electron transport.

The presence of pyridine nucleotide transhydrogenase in the mitochondrial subfragments supports the suggestion of Kaplan *et al.* (20) that the enzyme may link the TPN-reducing dehydrogenases with the DPN-linked system of phosphorylating electron transport. Since the digitonin subfragments contain the complete phosphorylating respiratory chain and only a few dehydrogenases, they can be considered as small subunits of the mitochondrial membrane containing only those enzymes which are closely associated with one another functionally. Ball and Cooper (21) have also demonstrated that transhydrogenase is tightly bound to particles from heart mitochondria and Kielley and Bronk (24) have found transhydrogenase activity in rat liver mitochondrial fragments prepared by sonic vibration. Thus the presence of the transhydrogenase in these integrated systems in a bound form supports the concept that the transhydrogenase has a specific function in the respiratory chain.

Intact liver mitochondria contain 30 to 40 moles of pyridine nucleotide per mole of cytochrome *a*, whereas the digitonin fragments contain less than one mole of rather specifically bound DPN, reactive with β-hydroxybutyric dehydrogenase, but not with malic dehydrogenase (17). This bound DPN has a much higher "turnover" than the total DPN pool in intact mitochondria (22). The possibility exists that pyridine nucleotide transhydrogenases associated with the electron transport system may link a large pool of free DPNH in the mitochondria with the

specifically bound DPN on the phosphorylating respiratory assembly in the membrane. Kaplan *et al.* (25) have demonstrated that the TPN–DPN transhydrogenase of beef heart also transfers hydrogen from DPNH to DPN^+. Experiments are in progress to determine whether rat liver mitochondria and the digitonin fragments prepared from them are also capable of carrying out this type of transhydrogenation.

SUMMARY

Difference spectra of phosphorylating subfragments of rat liver mitochondria, prepared by treatment with digitonin, were recorded under a variety of conditions. Cytochromes *a*, a_3, *b*, and *c*, and flavoprotein were identified as part of the respiratory chain in these particles. These carriers are completely reduced by D(−)-β-hydroxybutyrate under anaerobic conditions. Bound diphosphopyridine nucleotide could not be directly measured in the difference spectrum, but was determined by an enzymatic assay. The relative concentrations of cytochromes *a*, a_3, *b*, and *c*, flavoprotein and diphosphopyridine nucleotide in the subfragments were found to be 1.0:1.0:0.7:1.8:3.0:0.3, respectively. These proportions are very similar to those observed in intact rat liver mitochondria, with the exception of diphosphopyridine nucleotide, indicating that the phosphorylating fragments prepared with digitonin suffer very little damage to the respiratory assemblies during isolation. Some of the cytochrome *b* present in the particles appears to be "dislocated" from the respiratory chain. The fragments also contain a pigment which is reducible by hydrosulfite but not by β-hydroxybutyrate; it does not appear to lie directly on the respiratory chain. The digitonin particles also contain a TPN–DPN pyridine nucleotide transhydrogenase which is inhibited by L-thyroxine. A special role of bound DPN and bound pyridine nucleotide transhydrogenase in phosphorylating electron transport is discussed.

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REFERENCES

1. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **219**, 489 (1956).
2. Devlin, T. M., and Lehninger, A. L., *J. Biol. Chem.*, **219**, 507 (1956).
3. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **219**, 519 (1956).
4. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **224**, 547 (1957).
5. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **224**, 561 (1957).
6. Wadkins, C., and Lehninger, A. L., *J. Am. Chem. Soc.*, **79**, 1010 (1957).
7. Chance, B., and Williams, G. R., *J. Biol. Chem.*, **217**, 395 (1955).
8. Rabinowitz, M., and DeBernard, B., *Biochim. et Biophys. Acta*, **26**, 22 (1957).
9. Crane, F. L., and Glenn, J. L., *Biochim. et Biophys. Acta*, **24**, 100 (1957).
10. Devlin, T. M., and Lehninger, A. L., *J. Biol. Chem.*, **233**, 1586 (1958).
11. Mehler, A. H., *Science*, **120**, 1043 (1954).
12. Sandell, E. B., *Colorimetric determination of traces of metal, Vol. III*, Interscience Publishers, Inc., New York, 1944.
13. Chance, B., and Williams, G. R., *J. Biol. Chem.*, **217**, 409 (1955).
14. Estabrook, R. W., and Mackler, B., *J. Biol. Chem.*, **224**, 637 (1957).
15. Estabrook, R. W., *J. Biol. Chem.*, **230**, 735 (1958).
16. Ball, E. G., and Cooper, O., *J. Biol. Chem.*, **226**, 755 (1957).
17. Gamble, J. L., Jr., and Lehninger, A. L., *J. Biol. Chem.*, **223**, 921 (1956).
18. Polis, B. D., and Shmukler, H. W., *J. Biol. Chem.*, **227**, 419 (1957).
19. Hülsmann, W. C., Elliott, W. B., and Rudney, H., *Biochim. et Biophys. Acta*, **27**, 663 (1958).
20. Kaplan, N. O., Swartz, M. N., Frech, M. E., and Ciotti, M. M., *Proc. Natl. Acad. Sci. U. S.*, **42**, 481 (1956).
21. Ball, E. G., and Cooper, O., *Proc. Natl. Acad. Sci. U. S.*, **43**, 357 (1957).
22. Lehninger, A. L., Wadkins, C. L., Cooper, C., Devlin, T. M., and Gamble, J. L., Jr., *Science*, **128**, 450 (1958).
23. Chance, B., *Nature*, **169**, 215 (1952).
24. Kielley, W. W., and Bronk, J. R., *J. Biol. Chem.*, **230**, 521 (1958).
25. Kaplan, N. O., Colowick, S. P., Zatman, L. J., and Ciotti, M. M., *J. Biol. Chem.*, **205**, 31 (1953).

Evidence Concerning the Mechanism of Adenosine Triphosphate Formation by Spinach Chloroplasts*

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It was recently observed that electron transport is coupled to phosphorylation in spinach chloroplasts exposed to light. The reduction of ferricyanide in the Hill reaction permits a simultaneous esterification of inorganic phosphate (1). At the same time, Arnon *et al.* (1) observed that the rate at which ferricyanide is reduced may be doubled by the presence of phosphorylating reagents. In our experience, the rate of ferricyanide reduction may be increased up to 3½ times if ATP is formed simultaneously (2). To account for this phenomenon of coupling, a working hypothesis was suggested (2) based on the widely described schemes for oxidative phosphorylation in mitochondria (3). The scheme is shown below, modified only by reversing the order of the last two reactions, for reasons to be presented in this paper:

$$\text{I. } H_2O + X + Y \rightarrow X\cdot H + Y\cdot O$$

$$\text{II. } Y\cdot O \rightarrow Y + \tfrac{1}{2}O_2$$

$$\text{III. } X\cdot H + I + A \rightarrow X + AH + I^*$$

$$\text{IV. } AH + Fe(CN)_6^{-3} \rightarrow A + H^+ + Fe(CN)_6^{-4}$$

$$\text{V. } I^* + ADP \rightarrow I \backsim ADP$$

$$\text{VI. } I \backsim ADP + P_i{}^1 \rightarrow ATP + I$$

In the reactions postulated above, $X\cdot H$ and $Y\cdot O$ stand for the primary reduced and oxidized products resulting from the cleaving of water, and AH is that component which reduces ferricyanide. Electrons are transferred from $X\cdot H$ to A, before the reduction of ferricyanide by this mechanism. The transfer from $X\cdot H$ to A requires the presence of a component called I, and is inhibited by the presence of the activated form I^*. Continued electron flow requires the removal of I^* and the regeneration of I, and this regeneration is accomplished by transferring the energy of I^* to a high energy phosphate bond.

Several tests of the above postulates are suggested by previous work on mitochondrial phosphorylations. For instance, one may determine whether arsenate will replace phosphate in stimulating electron flow without leading to formation of ATP (4). Secondly, the exchange reaction between P_i and the terminal phosphate of ATP could be studied (5, 6) to determine whether Reactions V and VI operate reversibly. Finally, the previously observed inhibition of electron transport in this system by ATP (2) has been explored further in terms of the reactions postulated.

EXPERIMENTAL

Preparation of chloroplasts from spinach was described previously (7). Chloroplasts were washed once in 0.40 M sucrose, 0.05 M Tris, 0.01 M KCl buffered at pH 7.8. Ferricyanide reduction was determined by direct observation of the reaction mixture at 400 mμ (2). The reaction mixture for ferricyanide reduction contained 40 μmoles of Tris at pH 7.8, 70 μmoles of NaCl, 1.5 μmoles of ferricyanide, and approximately 0.030 mg. of chlorophyll. Further additions are noted separately. Illumination was for 2 minutes at 25° (room temperature), aerobically, unless otherwise indicated.

ATP^{32} was prepared by photosynthetic phosphorylation with phenazine methosulfate as the oxidation-reduction cofactor (7) with the use of ADP and an excess of carrier phosphate plus P^{32}. It was isolated from the acid-denatured reaction mixture by adsorption on acid-washed Norit (4), and the charcoal was washed three times with distilled water. The ATP^{32} was eluted with NH_4OH-ethanol, precipitated as the barium salt, and redissolved with K_2SO_4. The absorption curve of the product fitted that of pure ATP closely, and the amount determined from the absorption at 260 mμ agreed with that expected from the specific activity of the original phosphate.

In the exchange studies, ATP was separated from P_i by adsorption on acid-washed Norit after the reaction was over. Labeled PO_4 was removed from the charcoal by hydrolysis in 1.0 N HCl at 100° for 10 minutes (4).

RESULTS

Ferricyanide reduction is stimulated by both arsenate and phosphate (Fig. 1). With Mg present, but no ADP, phosphate causes about a 20 per cent stimulation (*cf.* reference 2). However, with both Mg and ADP added, phosphate produces up to a 3-fold stimulation of the rate of reduction. Arsenate behaves like phosphate in the presence of ADP and Mg, but does not share the effect of phosphate in the absence of ADP. The response to increasing concentrations of arsenate is very similar to that with increasing amounts of phosphate, but the final rate with phosphate is always a little higher than that with arsenate. The difference between the two probably reflects the basal stimulation by phosphate in the absence of ADP, which does not occur with arsenate.

Also to be noted in Fig. 1 is an inhibition of the control rate

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[1] The abbreviations used are: P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

by ADP. Thus the rate without ADP is 250, and with ADP is 160, when neither phosphate nor arsenate is present.

It is evident from Fig. 1 that arsenate stimulation is completely dependent upon ADP. This is explored further in Figs. 2, *A* and *B*, which compare the response to increasing concentrations of ADP when either phosphate or arsenate is present. Fig. 2*A* shows the inhibition by low concentrations of ADP noted previously; this is due to a very rapid conversion to ATP, which inhibits the reduction during the remainder of the time that the chloroplasts are in the light (2). In the presence of arsenate no inhibition is seen, presumably because it interferes with ATP formation (see Fig. 6).

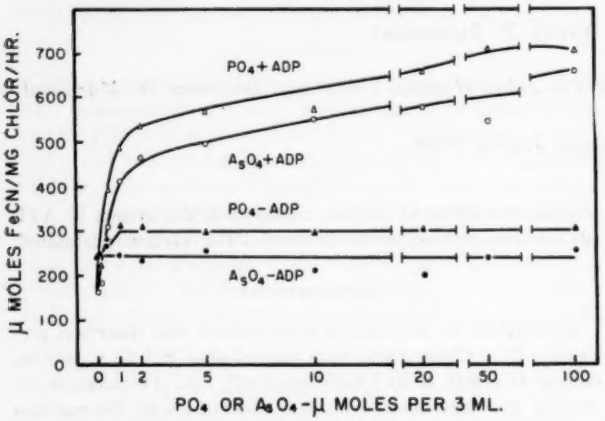

FIG. 1. Stimulation of ferricyanide reduction by phosphate esterification, and the substitution of arsenate for phosphate. The reaction mixtures contained 10 μmoles of $MgCl_2$ in addition to the basal components. The reaction mixtures represented by the two curves on top also contained 2 μmoles of ADP; those of the two on the bottom did not. Arsenate or phosphate as indicated.

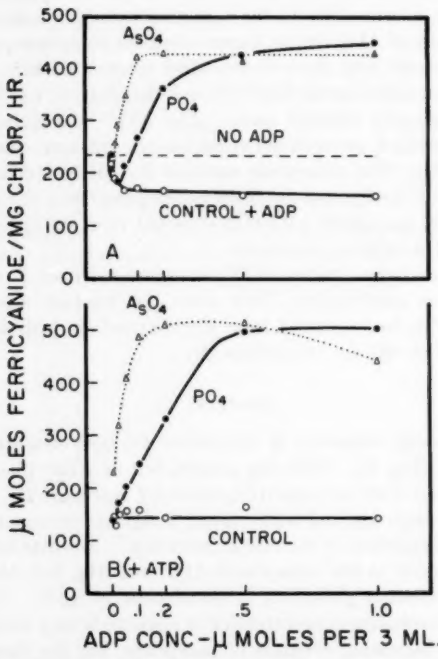

FIG. 2. Response of ferricyanide reduction to increasing concentrations of ADP, with phosphate or arsenate. Arsenate 25 μmoles or phosphate 25 μmoles, as indicated, and 10 μmoles of $MgCl_2$ present in all reaction mixtures. *A*. No ATP present; note the inhibition by low concentrations of ADP when phosphate is present but not when arsenate is present. *B*. 0.2 μmole of ATP added to all reactions; low concentrations of ADP do not give any extra inhibition.

In order to compare the ADP concentration curves for arsenate and phosphate directly without this complication, 0.2 μmole of ATP was added to all reaction mixtures in Fig. 2*B*. The initial rate with arsenate and ATP but with no ADP present is 50 per cent higher than that with phosphate and ATP. This could easily be due to a small amount of ADP contaminating the ATP used.

The major point seen in both Fig. 2*A* and 2*B* is that arsenate stimulation is saturated by 0.1 μmole of ADP; however, phosphate stimulation is not saturated until 0.5 μmole of ADP is provided. The ineffectiveness of lower ADP concentrations in phosphate stimulation is due to an early cessation of the stimulation. When a limiting concentration of ADP is used (Fig. 3), the stimulated rate with phosphate terminates at 1.5 minutes, presumably due to a complete conversion of ADP to ATP. Arsenate stimulation, on the other hand, continues until the chloroplasts lose activity (the time scale in Fig. 3 represents actual exposure to light; extra time needed to determine optical densities at the recorded points means that the chloroplasts had actually been exposed to 25° for 5 to 10 minutes by the time the last point was recorded). The initial reaction rates are the same for arsenate and phosphate, however.

The most likely interpretation of the time course experiment is that arsenate forms an unstable complex with ADP. Thus in the presence of arsenate ADP is regenerated fairly rapidly, and the ADP concentration is unchanged. If phosphate is present, however, the phosphate-ADP complex (*e.g.* ATP) is stable. This interpretation is confirmed by the experiment shown in Fig. 4: the same series of ADP concentrations was tested, but each reaction mixture also contained an excess of glucose and hexokinase. The added enzyme regenerates ADP from ATP artificially, and now phosphate shows the same response to low concentrations of ADP that arsenate does.

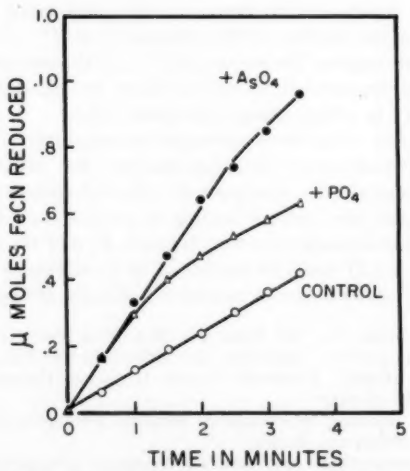

FIG. 3. Time course of reduction with a limiting amount of ADP. Arsenate or phosphate 25 μmoles; $MgCl_2$, 10 μmoles; and 0.25 μmole of ADP in each cuvette.

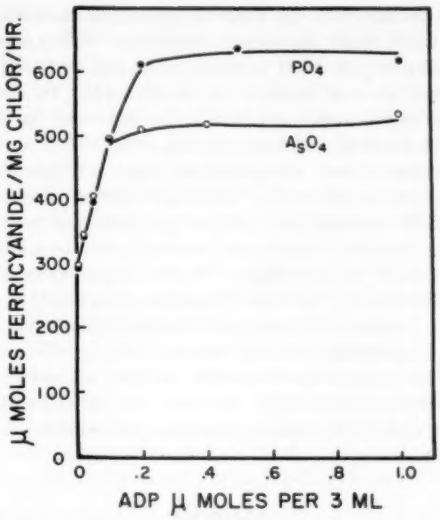

FIG. 4. Equivalent response of phosphate and arsenate to ADP, when ADP is regenerated artificially. Conditions as in Fig. 2A, except for the additions of 40 μmoles of glucose and an excess of crystalline hexokinase to each cuvette.

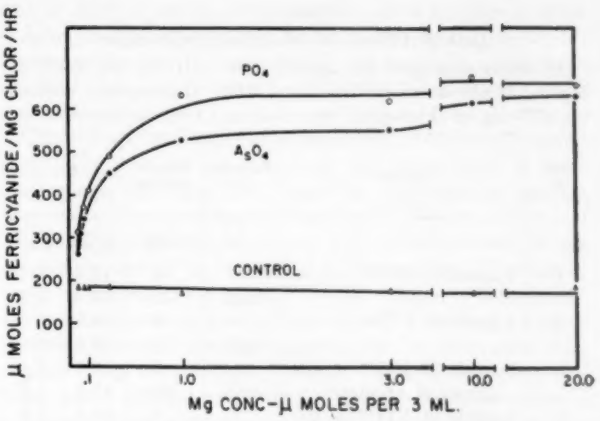

FIG. 5. Effect of Mg concentration on the stimulation of reduction by phosphorylation. All cuvettes contained 2.0 μmoles of ADP and 25 μmoles of phosphate or arsenate when present.

Stimulation by arsenate shows the same requirements for magnesium that the stimulation by phosphate does (Fig. 5).

Since arsenate can replace phosphate for the stimulation of the Hill reaction, it might be expected to inhibit ATP formation from ADP and phosphate. This inhibition does occur, and when the data are plotted according to the Lineweaver-Burk formulation (Fig. 6), it can be seen that arsenate is strictly competitive with phosphate. This was tested, as shown, both in the Hill reaction phosphorylation with ferricyanide as the oxidant and in the cyclic phosphorylation with phenazine methosulfate as the cofactor. It is possible to calculate an apparent K_m for phosphate of 5.4×10^{-4} M in the ferricyanide experiment, and 8.3×10^{-4} M in the cyclic phosphorylation experiment. The corresponding K_i values for arsenate are 6.7×10^{-4} M and 14.2×10^{-4} M. These values were obtained with different preparations of chloroplasts.

At least some of the ATPase activity of mitochondria has been considered to involve a part of the reactions of ATP formation acting reversibly, and has been used as a means for investigating the mechanism of oxidative phosphorylation (8–11). However, we have never been able to observe appreciable breakdown of ATP by chloroplasts (see Fig. 1 of reference 7, for instance). This is seen again in Table I, where ATP^{32} was incubated with chloroplasts under normal reaction conditions, both in the light and in the dark. The addition of 100 times as much arsenate as ATP^{32} still does not induce any significant breakdown of ATP, either in light or in darkness. In addition we have not seen any ATPase activity with chloroplasts uncoupled either by dilution in sodium chloride or by treatment with ammonium ions (12), nor in the presence of inhibitors such as trichlorophenolindophenol dye or *p*-chlorophenyldimethylurea (13).

Not only does it appear as if ATPase activity is missing from chloroplasts, but there is also no detectable exchange between P_i and ATP. Table II shows one out of a number of experiments in which either labeled inorganic phosphate and unlabeled ATP, or labeled ATP and unlabeled P_i, were supplied to chloroplasts in either the light or the dark. The "reaction" conditions in

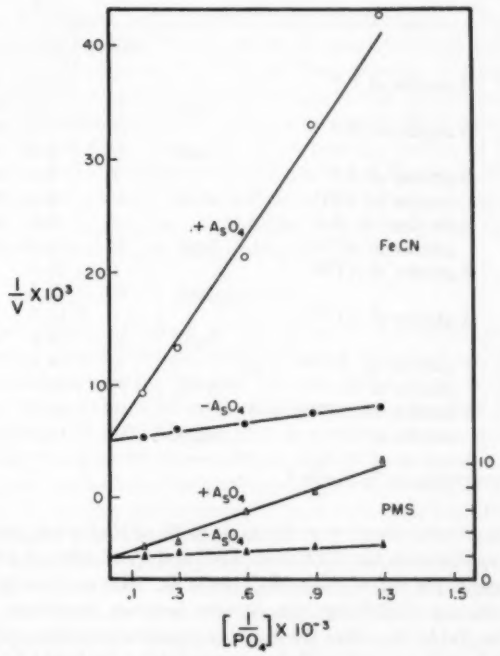

FIG. 6. Inhibition of ATP formation by arsenate, competitive with phosphate. Upper curves: 5 μmoles of ferricyanide present as the Hill oxidant; 3 μmoles of ADP, 5 μmoles of $MgCl_2$ and 0.047 mg. of chlorophyll present in a total volume of 1.5 ml. Arsenate, when present, represents 10 μmoles. Reaction was run under N_2, at 15° for 5 minutes. Bottom curves: 0.1 μmoles of phenazine methosulfate were substituted for the ferricyanide, chlorophyll was 0.026 mg. per flask and the reaction time was 3 minutes. Other conditions are the same as for the upper curves. ATP formation was measured using radioactive phosphate; activities shown are the reciprocals of μmoles of ATP^{32} formed per mg. of chlorophyll per hour; phosphate concentration is the reciprocal of the final molarity. The values on the ordinate to the left refer to the ferricyanide experiment, those to the right refer to the phenazine methosulfate experiment.

TABLE I

Lack of ATPase in chloroplast preparations

All flasks contained (in μmoles): Tris, pH 7.8, 20; NaCl, 35; $MgCl_2$, 5; phenazine methosulfate, 0.05. Chloroplasts containing 0.070 mg. of chlorophyll were used in a total volume of 1.5 ml.

Flask No.	Additions	Illumination	Time of reaction	Activity	
				ATP^{32}	P_i^{32}
			minutes	*c.p.m.* $\times 10^{-3}$	*c.p.m.* $\times 10^{-3}$
1	1 μmole of ATP^{32}		0	14.3	1.7
		dark	10	13.4	1.4
2	1 μmole of ATP^{32}		0	13.5	1.3
		light	10	13.9	0.9
3	1 μmole of ATP^{32} + 100 μmoles of arsenate		0	14.3	1.0
		dark	10	13.6	1.7
4	1 μmole of ATP^{32} + 100 μmoles of arsenate		0	14.1	0.6
		light	10	14.1	1.0

TABLE II

*Lack of P_i-ATP exchange in chloroplast preparations**

Flask No.	Additions	Illumination	Time	Activity	
				ATP	P_i
			minutes	*c.p.m.* $\times 10^{-3}$	*c.p.m.* $\times 10^{-3}$
1	5 μmoles of P_i^{32}		0	0.1	21.0
		dark	10	0.2	19.6
2	5 μmoles of P_i^{32}		0	0.1	19.8
		light	10	0.2	19.4
3	5 μmoles of P_i^{32} + 5 μmoles of ATP		0	0.1	18.8
		dark	10	0.2	18.9
4	5 μmoles of P_i^{32} + 5 μmoles of ATP		0	0.0	19.7
		light	10	0.8	19.3
5	5 μmoles of ATP^{32}		0	14.5	1.4
		dark	10	12.6	1.6
6	5 μmoles of ATP^{32}		0	13.5	1.3
		light	10	13.9	0.9
7	5 μmoles of ATP^{32} + 5 μmoles of P_i		0	14.4	1.4
		dark	10	12.9	1.6
8	5 μmoles of ATP^{32} + 5 μmoles of P_i		0	13.5	1.3
		light	10	12.3	1.7

* Conditions as in Table I.

the experiment shown were the same as those that would permit ATP synthesis in the light from ADP and P_i at rates of 300 to 600 μmoles per mg. of chlorophyll per hour. However, no significant amount of exchange can be seen between phosphate and ATP in Table II, within the limits of experimental error (which are at least 5 per cent in these experiments). It should be emphasized that, in order to increase the chances of detecting any ATPase or exchange reactions, in this experiment the amount of chloroplasts (0.070 mg. of chlorophyll per 1.5 ml.) and the length of time (10 minutes) were twice those usually used, and formation of ATP would certainly have gone to completion. In other experiments both the normal amount of chloroplasts and up to 4 times as much as usual were used. In some cases ATP^{32} formation from ADP and P^{32} was measured in a control flask, and this was always easily detectable by the methods used.

In mitochondria the exchange reaction may be inhibited by the presence of some oxidation-reduction dyes, presumably because they affect the oxidation-reduction level of an internal electron carrier (14). In Table II phenazine methosulfate was present which might have been an inhibitor. Other experiments were therefore performed in which phenazine methosulfate was eliminated, or was replaced by ferricyanide. In every case duplicate flasks were incubated in the light and in the dark. Again, no exchange reaction could be seen.

Under specialized circumstances with mitochondrial subparticles the phosphate-ATP exchange reaction does not occur unless ADP is added (15). All of the attempted exchange experiments described above were repeated, with the addition of 1 μmole of ADP to each flask. In these experiments the ATP was labeled initially, and the exchange reaction would have led to a loss of radioactivity from the charcoal adsorbable fraction. Again, no significant exchange reaction could be observed under any of the various conditions: *i.e.* whether electron transport was occurring or not (light or dark), and whether oxidation-reduction dyes (ferricyanide, phenazine methosulfate) were present or not.

TABLE III

ATP effect on Hill reactions

Chloroplast concentrations: 0.030 mg. of chlorophyll in fresh chloroplasts for ferricyanide reduction; 0.021 mg. of chlorophyll in activated chloroplasts reducing ferricyanide; and 0.012 mg. of chlorophyll in fresh chloroplasts reducing indophenol dye. Chloroplasts were activated by dilution in NaCl. Reaction time was 2 minutes for ferricyanide reduction, and 30 seconds for dye reduction.

Chloroplasts	Electron acceptor	Control rate*	Rate with ATP (as percentage of control)		
			0.05 μmole	0.1 μmole	0.2 μmole
			%	%	%
Fresh	ferricyanide	207	75	77	76
Fresh	indophenol dye	850	96	95	105
Activated	ferricyanide	770	100	100	99

* Control rates expressed as microequivalents of oxidant reduced per mg. of chlorophyll per hour.

TABLE IV

Reversibility of ATP inhibition of ferricyanide reduction

Reaction mixtures contained 0.028 mg. of chlorophyll per 3.0 ml. before washing, and 0.023 mg. of chlorophyll after washing. One-half the chloroplasts were stored in buffer containing 0.5 μmole of ATP per ml., as shown in the first column, and one-half were stored in buffer without ATP. Aliquots from both tubes were assayed for ferricyanide reduction with and without the addition of 0.2 μmole of ATP/3.0 ml. After this initial determination both sets of chloroplasts were diluted with 7 times the original volume of buffer, centrifuged, and resuspended in fresh buffer, in both cases without ATP present. They were then assayed again, with and without the addition of ATP to the reaction mixture.

Chloroplast storage	Activity* before washing		Activity after washing	
	−ATP	+ATP	−ATP	+ATP
−ATP	227	189	222	177
+ATP	165	167	208	180

* All activities shown are μmoles of ferricyanide reduced per mg. of chlorophyll per hour.

The failure of ATP to be broken down or even exchanged by chloroplasts makes it necessary to reconsider the inhibition of ferricyanide reduction by ATP (2). ATP in the experiment shown in Table III causes a 25 per cent inhibition of ferricyanide reduction by chloroplasts, but does not affect the reduction of trichloroindophenol dye. Also it does not affect the reduction of ferricyanide by chloroplasts that have been "uncoupled" by dilution in 0.35 M NaCl at pH 6.0.[2] Thus the inhibition can be seen only in chloroplasts where coupled phosphorylation is possible.

Attempts were made to observe any possible binding of small amounts of radioactive phosphate from ATP^{32} by large quantities of chloroplasts, but no such binding could be seen.

The inhibition by ATP appears to be reversible, to a large extent (Table IV). In this experiment, chloroplasts stored in ATP were not inhibited by extra ATP added to the reaction mixture. However after these were diluted, recovered by centrifugation, and resuspended in fresh buffer, their activity was largely restored, and they were again inhibitable by ATP.

DISCUSSION

It is evident that arsenate can substitute for phosphate in the reactions which normally lead to ATP formation. On the basis of Figs. 1 to 5, an unstable arsenate-ADP complex may be postulated. In accordance with the mechanism suggested in the introduction, the reactions in which arsenate and ADP participate can be formulated as:

$$\text{V. } I^* + ADP \rightarrow I \backsim ADP$$

$$\text{VII. } I \backsim ADP + AsO_4 \rightarrow I + (ADP \backsim AsO_4)$$

$$\text{VIII. } (ADP \backsim AsO_4) \xrightarrow{H_2O} ADP + AsO_4$$

The only net change from Reactions V, VII, and VIII is the conversion of I* to I. This permits electron flow to proceed rapidly, according to the proposed mechanism.

It is necessary to place the order of the reactions of ADP and AsO_4 in the sequence shown above in order to avoid postulating a stable high energy AsO_4 compound as an intermediate. In view of the competitive relationship between phosphate and arsenate, the same order of addition may be postulated for phosphate and ADP in the actual formation of ATP. Thus the order of Reactions V and VI suggested in the introduction depends entirely on the unlikelihood of a stable high energy arsenate intermediate. This sequence is the reverse of that shown recently in oxidative phosphorylation (16).

Stimulation of electron transport, and inhibition of phosphorylation in mitochondria by arsenate was studied by Crane and Lipmann (4). In their work about 50 per cent of the stimulation was obtained in the absence of added ADP, and the remaining 50 per cent required the addition of ADP. Their observations were complicated by reduction of the arsenate to arsenite, which then exerted a secondary inhibition on the oxidation of their substrate, α-ketoglutarate. No similar complications are apparent in the chloroplast system.

According to the scheme suggested, arsenolysis of ATP would be expected provided only that Reaction VI, the actual formation of ATP, is appreciably reversible. In a number of other systems phosphate bonds may be broken by arsenolysis (17–19, for example). Since no arsenolysis is observed with chloroplasts (Table I), we may propose that Reaction VI is essentially irreversible.

Confirmatory evidence for an irreversible step in ATP formation comes from the absence of any ATPase activity in these chloroplasts, and from the absence of any exchange reaction between ATP and inorganic phosphate.

Inhibition of the Hill reaction by ATP therefore cannot be due to a simple mass action reversal of ATP formation. The significance of the inhibition by ATP is still obscure. "Uncoupled" electron transport, such as the reduction of trichlorophenolindophenol dye, or the reduction of ferricyanide by salt-treated chloroplasts (2) is not inhibited by ATP. This suggests the possibility that a different mechanism of electron transport occurs in uncoupled chloroplasts than that found in coupled chloroplasts.

Coupled phosphorylation by chloroplasts is distinctly different in this respect than that seen in mitochondria, where ATPase activity and exchange reactions are abundant (8–11, 14–16). In photosynthetic phosphorylation by particles from species of *Chromatium*, 60 per cent as much P_i^{32} could be incorporated into ATP by exchange as by net ATP formation (20, Table IX), indicating either a very active exchange reaction, or else an active ATPase reaction followed by resynthesis of ATP. Photophosphorylation by chromatophores of *Rhodospirillum rubrum* appears to be unaccompanied by ATPase activity (21) and in this case the mechanism might be similar to that of chloroplasts.

Lack of exchange in chloroplasts compared to its presence in these other systems means that some reaction leading to ATP formation has a different mechanism here. If the formation of ATP is irreversible *in vivo* as well as *in vitro*, it may mean that higher plant photosynthetic phosphorylation is to that extent more efficient than respiratory phosphorylation.

SUMMARY

Arsenate can substitute for phosphate in chloroplast photosynthetic phosphorylation, and also in the stimulation of ferricyanide reduction by the Hill reaction. The arsenate effect requires the addition of both magnesium and adenosine 5′-diphosphate, and it is suggested that an adenosine 5′-diphosphate-arsenate complex is formed but is rapidly hydrolyzed. It is concluded tentatively that addition of either arsenate or phosphate occurs after the formation of an intermediate high energy adenosine 5′-diphosphate complex.

No adenosine 5′-triphosphatase activity, arsenolysis of adenosine 5′-triphosphate, or exchange reaction between inorganic phosphate and adenosine 5′-triphosphate can be observed with these preparations. It is concluded that the last step in adenosine 5′-triphosphate formation, according to the proposed scheme, is an irreversible one.

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REFERENCES

1. Arnon, D. I., Whatley, F. R., and Allen, M. B., *Science*, **127**, 1026 (1958).
2. Avron, M., Krogmann, D. W., and Jagendorf, A. T., *Biochim. et Biophys. Acta*, **30**, 144 (1958).
3. Chance, B., and Williams, G. R., *Advances in Enzymol.*, **17**, 65 (1956).
4. Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201**, 235, (1953).

5. Lee, K.-H., and Eiler, J. J., *J. Biol. Chem.*, **203**, 705 (1953).
6. Boyer, P. D., Falcone, A. B., and Harrison, W. H., *Nature*, **174**, 401 (1954).
7. Jagendorf, A. T., and Avron, M., *J. Biol. Chem.*, **231**, 277 (1958).
8. Lardy, H. A., and Elvehjem, C. A., *Annual review of biochemistry, Vol. 14*, Annual Reviews, Inc., Palo Alto, California, 1945.
9. Hunter, F. E., Jr., in W. D. McElroy, and H. B. Glass (Editors), *Phosphorus metabolism, Vol. I*, Johns Hopkins Press, Baltimore, 1951, p. 297.
10. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **201**, 357 (1953).
11. Anfinson, C. B., and Kielley, W. N., *Annual review of biochemistry, Vol. 23*, Annual Reviews, Inc., New York, 1954, p. 17.
12. Krogmann, D. W., Jagendorf, A. T., and Avron, M., *Plant Physiol.*, in press.
13. Jagendorf, A. T., and Avron, M. *Arch. Biochem. Biophys.*, in press.
14. Low, H., Siekevitz, P., Ernster, L., and Lindberg, O., *Biochim. et Biophys. Acta*, **26**, 225 (1957).
15. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **224**, 561 (1957).
16. Wadkins, C. L., and Lehninger, A. L., *Federation Proc.*, **17**, 329 (1958).
17. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
18. Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **170**, 147 (1947).
19. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184**, 769 (1950).
20. Newton, J. W., and Kamen, M. D., *Biochim. et Biophys. Acta*, **25**, 462, (1957).
21. Frenkel, A., *J. Am. Chem. Soc.*, **76**, 5568 (1954).

Hydrogen Oxidation by *Clostridium kluyveri*

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In 1952, Korkes (1) reported that cell-free extracts of *Clostridium kluyveri* were capable of reducing pyridine nucleotides with hydrogen. Subsequent investigations have shown that a heat-stable cofactor of undetermined nature present in boiled cell extracts was required (2). Pyridine nucleotide reduction with hydrogen has since been demonstrated in a number of other microorganisms (3–5).

The present study was undertaken to explore in greater detail the alternative routes of hydrogen oxidation in *C. kluyveri*. Particular attention was paid to a comparison of the requirements for pyridine nucleotide, flavin nucleotide, and dye reduction. The available data suggest that the unknown cofactor does not function in the activation of hydrogen but acts between an activated hydrogen intermediate and pyridine nucleotide. Additional evidence is also presented showing that more than one enzyme is required for pyridine nucleotide reduction with hydrogen. A preliminary report of these results has been published (6).

EXPERIMENTAL

Materials—The following chemicals were purchased from commercial sources. DPN and TPN (Pabst Laboratories), FAD (Sigma), GSH (Nutritional Biochemicals), triphenyl tetrazolium chloride (Schwarz Laboratories), methylene blue (Difco Laboratories), benzyl viologen (Mann Laboratories), Safranin O (Coleman and Bell), indigo disulfonate and 2,6-dichlorophenol indophenol (Fisher Scientific), triethanolamine (Eastman), crystalline lactic dehydrogenase (Worthington Biochemical). Synthetic FAD was kindly provided by Dr. N. O. Kaplan.

Preparation of Enzyme—Dried cells of *C. kluyveri* were obtained by the method of Stadtman and Barker (7). Dialyzed cell-free extracts were prepared by a slight modification of Korkes' procedure (2). A gm. of dried cells was added to 15 ml. of phosphate-GSH buffer (1.5 ml. of 0.1 M KH_2PO_4, 1.5 ml. of 0.1 M GSH, 12 ml. of H_2O, final pH 7.5) which had been thoroughly flushed with hydrogen. After 4 hours of incubation under hydrogen with shaking at 30°, the suspension was centrifuged at 12,000 × *g* for 20 minutes and the precipitate discarded. The supernatant solution was dialyzed in the cold against 1 l. of 0.01 M potassium phosphate-0.001 M GSH, pH 7.5, under hydrogen for 20 hours. Before dialysis, the buffer was flushed vigorously with a stream of hydrogen. Dialyzed extracts were stored under hydrogen at −15°.

Preparation of Cofactor—Boiled cell extracts were prepared by adding 5 gm. of dried *C. kluyveri* cells to 150 ml. of distilled water preheated to 90°. The temperature was maintained between 90 and 95° and the mixture stirred manually for 10 minutes. After cooling to 0°, the boiled extract was centrifuged and the precipitate discarded. The supernatant solution was used as a crude cofactor source and for preliminary purification of the cofactor.

Activity Determination—Reproducible results were obtained only under rigorously anaerobic conditions. Incubations were performed in 13 × 100-mm. test tubes which initially contained buffer, substrate, DPN, and cofactor (when added). A rapid stream of tank hydrogen or helium emanating from a 22-gauge, 5-inch-long, hypodermic needle was directed on the surface of the tube contents. After flushing for 1 minute, all labile and autoxidizable components (*e.g.* ferrous sulfate, GSH, lactic acid dehydrogenase, *C. kluyveri* extract) were rapidly added and the tubes stoppered simultaneously with removal of the hypodermic needle. This procedure assured nearly complete anaerobiosis, and if any dissolved oxygen did remain it was readily consumed by the powerful pyridine nucleotide oxidase present in *C. kluyveri*.

As reported previously, ferrous sulfate and GSH increase the rate of pyridine nucleotide reduction (2). In the present experiments a similar stimulation was observed when dye reduction was measured; ferrous sulfate and GSH were consequently routinely added to the standard reaction mixture.

DPN reduction was determined indirectly by coupling with lactic dehydrogenase and measuring the amount of pyruvate which disappeared under a hydrogen gas phase. The complete system contained 200 μmoles of triethanolamine buffer, pH 8; 3 μmoles of potassium pyruvate; 5 μmoles of $FeSO_4$; 3 μmoles of GSH; lactic dehydrogenase (sufficient to catalyze the oxidation of 12 μmoles of DPNH per minute under standard assay conditions (8)), and *C. kluyveri* extract. The last four components were added in the order mentioned under a gas stream as described above. The final volume was 1.5 to 1.8 ml. The reaction tubes were incubated for 15 minutes at 30° with shaking. The reaction was stopped with 0.2 ml. of 10 per cent perchloric acid, and after centrifugation the supernatant solution was analyzed for residual pyruvate according to the method of Friedemann and Haugen (9). All values were corrected for any pyruvate disappearing under a helium atmosphere.

When hydrogen oxidation by dye was measured, the complete system contained 200 μmoles of triethanolamine buffer, pH 8; 10 μmoles of triphenyl tetrazolium, 5 μmoles of $FeSO_4$; 3 μmoles GSH; and *C. kluyveri* extract; final volume 1.5 to 1.8 ml. The enzyme was again added last under a gas stream, and the tubes were stoppered and incubated for 15 minutes at 30° with shaking. At the end of the incubation period 0.2 ml. of 10 per cent perchloric acid was added, the insoluble reduced tetrazolium (formazan) was dissolved by addition of 5 volumes of ethanol, and the

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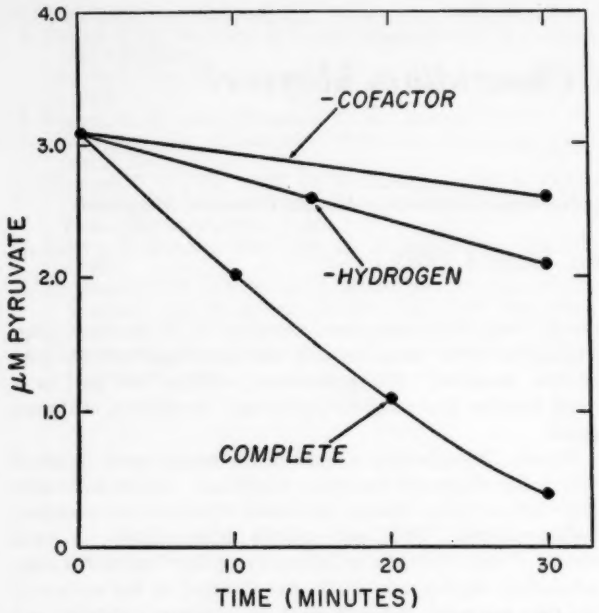

FIG. 1. Effect of cofactor on rate of pyruvate reduction with hydrogen. Pyruvate reduction was determined by the standard assay procedure; incubation time was varied as indicated on the abscissa.

TABLE I

Requirements for pyruvate and tetrazolium reduction

Reactant omitted	Pyruvate* reduced in 15 minutes		Tetrazolium reduced in 15 minutes	
	μmoles	μmoles	μmoles	μmoles
None	2.72	2.82	4.17	4.11
H_2(He)	0.84	0.79	0.55	
DPN	1.88		4.46	4.11
Cofactor	0.56	0.53		4.53
Lactic dehydrogenase	0.24			
Clostridium kluyveri extract	0.00		0.20	

* Pyruvate and dye reduction determined by standard assay procedure.

precipitated protein was removed by centrifugation. The color intensity of the supernatant solution was determined with a Klett colorimeter (No. 540 filter), and the amount of dye reduced was calculated from a standard curve. Corrections were made for any dye reduction occurring under helium.

Protein was determined by the method of Lowry *et al.* (10). Spectrophotometric determinations were made with a Beckman model DU or Cary recording spectrophotometer.

RESULTS

Cofactor Requirement for Pyridine Nucleotide Reduction—Freshly prepared extracts of *C. kluyveri* catalyze the reduction of pyridine nucleotides by hydrogen.[1] Anaerobic dialysis of the extract completely abolished this activity. Nearly complete restoration could be obtained by addition of boiled cell extract and representative results are shown in Fig. 1. The complete system, which contained dialyzed *C. kluyveri* extract, lactic dehydrogenase, boiled cell extract, and DPN, catalyzed a rapid disappearance of pyruvate when incubated under hydrogen. Substitution of helium for hydrogen, or omission of the cofactor, produced a marked decrease in the amount of pyruvate which disappeared. In the presence of boiled cell extract almost all the added pyruvate was reduced within 30 minutes and the rate remained essentially linear.

Effect of Cofactor on Dye Reduction—The possible involvement of the cofactor present in the boiled cell extract in dye reduction was investigated, since this has been the conventional method for determining hydrogenase activity. Triphenyl tetrazolium was employed in the present study, although similar results have been obtained with benzyl viologen, Safranin O, indigo disulfonate, methylene blue, and indophenol. Data obtained from duplicate determinations are presented in Table I. As expected, there is very little pyruvate or tetrazolium reduction when either hydrogen or dialyzed *C. kluyveri* extract is absent from an otherwise "complete" system. Addition of DPN is required for maximal pyruvate reduction and the appreciable pyruvate disappearance observed in the absence of added DPN is due to the large amounts of the latter present in the boiled cell extract. Only very slight pyruvate disappearance occurs when lactic dehydrogenase is omitted, showing that pyruvate reduction is, indeed, a reliable measure of DPN reduction with hydrogen.

In contrast, neither DPN nor the cofactor are required for tetrazolium reduction. Repeated attempts to show a cofactor or DPN requirement for dye reduction have been consistently negative. The significance of these results will be discussed subsequently.

Effect of Cofactor on Flavin Reduction—Extracts of *C. kluyveri* can also catalyze the rapid reduction of flavin nucleotides with hydrogen. To test the effect of cofactor on flavin reduction, a dialyzed extract was incubated with and without boiled cell extract in the main compartment of a modified Thunberg tube with a Beckman cuvette attached. After 2 minutes the optical density at 450 mμ attained a constant value and riboflavin monophosphate was added from the side arm. As shown in Fig. 2, the rate of riboflavin monophosphate reduction was essentially the same in the presence and absence of cofactor. This is analogous to the situation observed with dyes and it should be noted that, in all cases in which dye and flavin reduction were compared, qualitatively similar results have been obtained.

Number of Protein Components Involved in Pyridine Nucleotide and Dye Reduction—Comparison of dye and DPN reduction as catalyzed by different cell preparations of *C. kluyveri* provided support for the postulate that at least two enzymes are involved in pyridine nucleotide reduction by hydrogen (2). The data are presented in Table II. Extracts of cell lot B[2] are incapable

[1] Both DPN and TPN are reduced (2). In the present investigation TPN reduction was demonstrated by coupling with the triphosphopyridine nucleotide specific nitrate reductase from *Neurospora crassa* (11) and measuring the amount of nitrite formed under a hydrogen atmosphere. This assay was not routinely used in subsequent experiments because of the relative impurity of certain nitrate reductase preparations.

[2] Cell lots A and B refer to dried cell preparations of *C. kluyveri* made from cells which were grown in an identical manner but at different times and stored at −17° for approximately 2 years before use. The exact reason for the differences in activity described in the text is not yet clear. Stadtman and Barker (7) have previously observed that extracts prepared under similar conditions from different cell lots may show wide variability with respect to enzyme content.

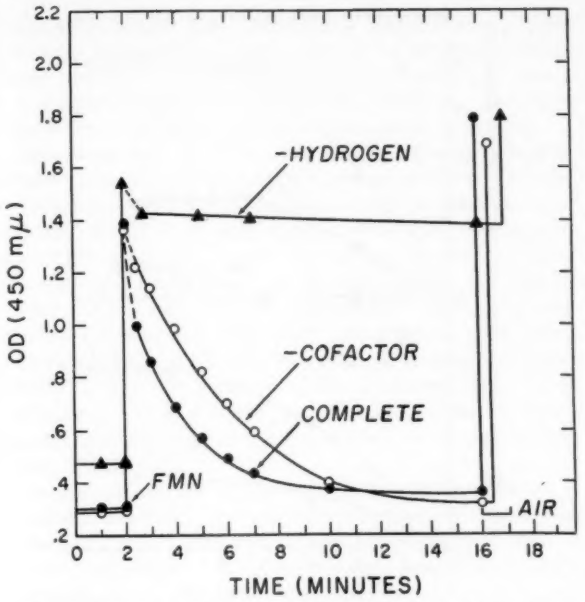

FIG. 2. Effect of cofactor on flavin reduction with hydrogen. The main compartment (Beckman cuvette) of a modified Thunberg tube contained initially 0.4 cc. of 1 M triethanolamine, pH 8; 0.3 ml. of boiled cell extract; and 2.01 ml. of H_2O. After flushing for 4 minutes with either hydrogen or helium, 0.1 ml. of 0.1 M $FeSO_4$ and 0.06 ml. of 0.1 M GSH were added and gassing continued for another minute. With a stream of gas playing on the surface of the tube contents, 0.1 ml. of *Clostridium kluyveri* extract was added, the tube stoppered and incubated at 30°. After 2 minutes, 0.03 ml. of 9.5×10^{-3} M riboflavin monophosphate was tipped in from the side arm. FMN represents riboflavin monophosphate.

TABLE II

Activity of cell lots A and B on pyruvate and tetrazolium reduction

Cofactor source	Enzyme source	
	Lot A	Lot B
	*μmoles pyruvate reduced**	
None	0.06	0.00
Lot B	0.93	0.02
	*μmoles tetrazolium reduced**	
None	6.66	7.74

* By 5.76 mg. of protein in 15 minutes. Pyruvate and tetrazolium reduction was determined by standard assay procedure.

of reducing DPN even when incubated with boiled cell extract prepared from the same source. That this boiled cell extract does contain the requisite cofactor is indicated by its ability to stimulate DPN reduction when incubated with extracts from cell lot A. Combined extracts from cell lots A and B were active, showing that the lack of activity in lot B preparations was not due to the presence of an inhibitor. Both extracts are equally capable of activating hydrogen as measured by tetrazolium reduction. (Although not indicated in the table both extracts were also capable of reducing riboflavin monophosphate with hydrogen.) A plausible interpretation of these results is that cell lot B, although possessing the enzymes required for the activation of hydrogen, lacks the protein component required for the subsequent reduction of DPN. In the case of cell lot B, this second enzyme may have been gradually destroyed during the long period of storage.[2]

The suggestion that two enzymes are required for DPN reduction was based on Korkes' observation (2) that some enzyme preparations consistently showed a parabolic increase in activity with increasing protein concentration in the presence of saturating amounts of heat stable factors. This phenomenon is not seen with freshly prepared extracts of cell lot A which, on the contrary, show an essentially linear relationship between activity (as measured either by tetrazolium or DPN reduction) and protein concentration. However, if these extracts are left at room temperature for 8 to 24 hours under a hydrogen atmosphere there is a loss (30 to 40 per cent) in the ability of the extract to reduce pyridine nucleotide, but dye-reducing activity remains nearly unchanged (5 per cent loss). Concurrent with this loss in activity towards pyridine nucleotide there is no longer a linear response to enzyme concentration but instead the rate of pyridine nucleotide reduction is a parabolic function of protein concentration (*Curve 3*, Fig. 3). Dye reduction, nevertheless, remains linear with respect to protein concentration (*Curve 1*, Fig. 3).

When the parabolic phenomenon is manifested the rate of pyridine nucleotide reduction is proportional to the square of

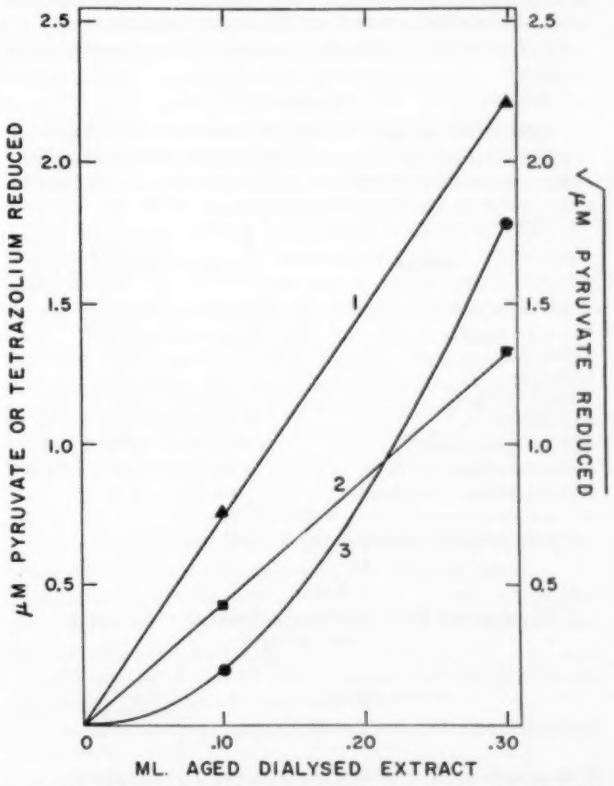

FIG. 3. Effect of protein concentration on pyruvate and tetrazolium reduction. Dialyzed *Clostridium kluyveri* extract was stored at room temperature (approximately 20°) for 14 hours under a hydrogen atmosphere and then assayed for activity according to standard assay procedure. Additional details in text.

extract concentration (in *Curve 2*, Fig. 3, the data of *Curve 3* have been recalculated to show the linear relation between the square root of the rate of DPN reduction and protein concentration). This dependence on the square of extract concentration suggests that at least two different reactions are rate-limiting for pyridine nucleotide reduction. One of these reactions was not rate-limiting in the fresh extract, which gave a linear response, but did become rate-limiting when the extract was aged. Since dye reduction continues to show a linear response with the aged extract, these results are taken as presumptive evidence that an additional enzyme not required for dye reduction is necessary for DPN reduction with hydrogen.

Role of Cofactor—Several experiments were performed to determine the function of the cofactor. DPN, when incubated with *C. kluyveri* extract, either in the presence or absence of boiled cell extract under standard assay conditions, could be recovered completely. This indicated that the cofactor did not act by preventing destruction of pyridine nucleotide.

Investigations by Peck and Gest (3) with *Escherichia coli* and *Clostridium pasteurianum* have shown that crude extracts could also reduce pyridine nucleotide when catalytic levels of benzyl viologen were present. Confirmatory results have been obtained with the *C. kluyveri* system, and the data in Table III show that benzyl viologen can replace the cofactor. However, substitution for the cofactor is not limited to this 1-electron dye but the 2-electron dye, Safranin O, can also function. Oxidation-reduction dyes with potentials appreciably higher than the pyridine nucleotide couple (−0.320) do not function.

Effect of FAD in Replacing Cofactor—The experiments with benzyl viologen and Safranin suggest that the cofactor present in the boiled cell extract acts by undergoing alternate reduction and oxidation and mediates the transfer of electrons between an activated hydrogen intermediate and pyridine nucleotide. This hypothesis is further supported by the observation that FAD is partially effective in replacing the cofactor.[3] Other naturally occurring materials which have been tested and which fail to replace the boiled cell extract include various pteridine derivatives, vitamins E and K, TPN, CoA, and lipoic acid. Crude TPN preparations from Sigma, yeast extracts from Sigma and Pabst, CoA residues from Pabst and Upjohn, and boiled cell extracts from *Micrococcus lactilyticus*[4] are all devoid of stimulatory activity; some of these preparations, however, do contain inhibitory materials which may have prevented detection of the cofactor.

The FAD-stimulated reduction of pyruvate by hydrogen also requires the presence of DPN and lactic dehydrogenase as indicated in Table IV. It is unlikely that the activity of FAD is due to a contaminant, since the natural and synthetic coenzymes

TABLE III

Effect of various dyes on pyruvate reduction with hydrogen

Pyruvate reduction was determined by the standard assay procedure except that the cofactor, when omitted, was replaced by either water or dye (final concentration 3.3×10^{-4} M).

Addition	E'_0	Pyruvate disappearing	Dye reduction in absence of cofactor
		μmoles	
None		0.25	
Cofactor		1.31	
Benzyl viologen	−0.359	2.27	+
Safranin O	−0.289	1.77	+
Indigo disulfonate	−0.125	0.10	+
Methylene blue	+0.011	0.00	+
Dichlorophenol indophenol	+0.217	0.14	*

* Nonenzymatic reduction due to GSH in extract.

TABLE IV

Requirements for FAD-stimulated reduction of pyruvate with hydrogen*

Reactant omitted	Pyruvate reduced (corrected for disappearance under helium)
	μmoles
None	1.27
FAD (0.12 μM)	0.31
DPN	0.00
Lactic dehydrogenase	0.00
Clostridium kluyveri extract	0.00

* Pyruvate reduction determined by standard assay procedure.

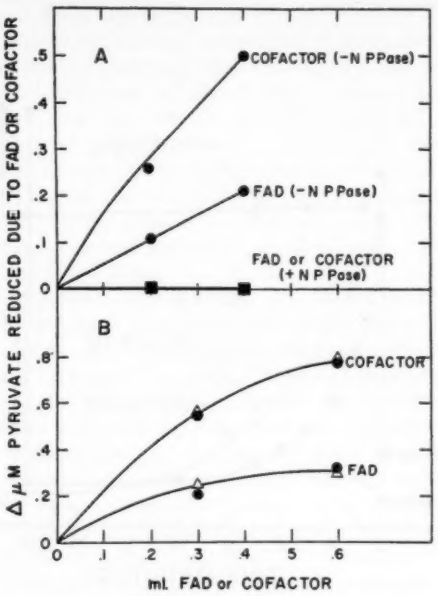

FIG. 4. In both experiments, FAD and boiled cell extract, buffered at pH 7.5, with equal extinctions at 450 mμ, were used (E_{450} = 0.86). Pyruvate reduction determined by standard assay procedure. *A*. Effect of nucleotide pyrophosphatase on stimulatory activity of FAD and cofactor. The FAD solution and boiled cell extract, 3 ml. each, were each incubated with 10 units of the pyrophosphatase for 4 hours at 30°. The reaction was stopped by placing the tubes in boiling water for 5 minutes. Control tubes were incubated without enzyme and nucleotide pyrophosphatase was added immediately before heat inactivation. *B*. Effect of ultraviolet illumination on stimulatory activity of FAD and cofactor. The FAD solution and boiled cell extract, 3 ml. each, were each illuminated at room temperature for 64 hours with an ultraviolet lamp (λ_{max} = 366 mμ) placed 0.5 inch from the tubes. Control tubes were covered with aluminum foil. At the end of this time period the extinction had decreased 63 and 47 per cent for the FAD and boiled cell extract, respectively. △, with ultraviolet; ●, without ultraviolet; NPPase, nucleotide pyrophosphatase.

[3] Previous attempts to demonstrate stimulation by FAD failed (6). This was due to the use of relatively impure preparations of FAD containing significant amounts of riboflavin monophosphate which inhibit the reaction.

[4] Kindly supplied by Dr. G. D. Novelli.

possess identical activities. Thus, 0.085 μmole of natural FAD from yeast (Sigma, 65 per cent pure) or the synthetic product (60 per cent pure) each stimulated the reduction of 0.61 μmole of pyruvate. Lumichrome, lumiflavin, riboflavin, and riboflavin monophosphate do not have any appreciable activity.

Relationship Between FAD and Cofactor—The available data indicate that FAD and the cofactor are not identical. The concentration (as estimated on the basis of 450 mμ absorption) of cofactor and of FAD required to produce the same rate of pyruvate reduction is much lower in the case of the cofactor. This is shown in Fig. 4. Not only are the saturation curves different but the maximal rate of pyruvate reduction is 1.5 times greater with the cofactor than with FAD. In a typical experiment, saturation levels of FAD and the cofactor stimulated the reduction of 0.93 and 1.51 μmoles of pyruvate, respectively. When both were present at saturation, 1.53 μmoles of pyruvate disappeared, indicating that the lower rate is not due to an inhibitor present in FAD.

The stimulatory activity of both FAD and the cofactor is completely abolished by nucleotide pyrophosphatase (12) as shown in Fig. 4*A*. This suggests that the active component in the boiled cell extract has a pyrophosphate bond and may be a dinucleotide.

Light inactivation studies have produced curious results. When neutral solutions of FAD or the boiled cell extract are illuminated for several days with either a tungsten or ultraviolet lamp, essentially no loss in stimulatory activity is observed, although the absorption at 450 mμ may have decreased as much as 60 per cent. The results are depicted in Fig. 4*B*. The results suggest that an intact isoalloxazine ring system may not be required for activity. Isolation of the active products of illuminated FAD is now in progress.

DISCUSSION

The present investigation of hydrogen oxidation by *C. kluyveri* has suggested the following main points. (*a*) Flavin reduction (as well as tetrazolium, methylene blue, indigo disulfonate, and indophenol reduction) does not require the participation of a heat stable cofactor which is necessary for pyridine nucleotide reduction. (*b*) The cofactor probably functions as an electron carrier between an activated hydrogen intermediate and DPN because it can be replaced by benzyl viologen and Safranin O. (*c*) FAD is partially effective in substituting for the cofactor. (*d*) At least two enzymes are involved in pyridine nucleotide reduction. (*e*) An enzyme not required for dye or flavin reduction is necessary for DPN reduction. These conclusions are illustrated in Diagram 1.

DIAGRAM 1

$$H_2 \rightarrow [H_2] \rightarrow \left.\begin{array}{l}\text{Cofactor}\\ \text{benzyl viologen}\\ \text{Safranin O}\\ \text{FAD}\end{array}\right\} \rightarrow \text{DPN}$$

riboflavin phosphate
indigo disulfonate
triphenyl tetrazolium
methylene blue
dichlorophenol indophenol

This scheme may not only describe hydrogen oxidation in *C. kluyveri* but may also operate in other organisms. Wittenberger and Repaske (5) have recently shown that unidentified cofactors are required for the reduction of DPN by freshly prepared extracts of *Hydrogenomonas eutropha*. Upon prolonged storage these extracts lose all ability to reduce pyridine nucleotides but can still reduce methylene blue with hydrogen.

However, the system described by Packer and Vishniac (13) in *Hydrogenomonas ruhlandii* differs from that of *C. kluyveri* in several interesting and significant features. (*a*) DPN reduction in *H. ruhlandii* is dependent on catalytic levels of riboflavin monophosphate. FAD, but not the monophosphate, is effective in the *C. kluyveri* system. (*b*) With *H. ruhlandii*, DPN was required catalytically for the reduction of benzyl viologen and methylene blue. No evidence has been obtained for a DPN requirement for dye reduction by *C. kluyveri*. Furthermore, Peck and Gest (3) have shown that benzyl viologen is necessary in catalytic amounts for DPN reduction by preparations of *E. coli* which contain little flavin and do not reduce riboflavin monophosphate with hydrogen. (*c*) *H. ruhlandii* could catalyze the evolution of hydrogen from DPNH. Korkes was unable to demonstrate such a reaction in *C. kluyveri*.

The fact that flavin nucleotides and dyes can be reduced in the absence of cofactor suggests that this cofactor does not function directly in the activation of hydrogen. These results are in agreement with those obtained by Krasna *et al.* (14), for the purified soluble hydrogenase of *Desulfovibrio desulfuricans*. With the use of the deuterium exchange method, these workers were unable to obtain any evidence for the participation of a cofactor in the activation of molecular hydrogen.

Numerous investigators have postulated that the reduction of pyridine nucleotides by hydrogen and the photolytic reduction of pyridine nucleotides may involve common reactions (15). Dr. A. San Pietro has kindly provided us with a sample of spinach photosynthetic pyridine nucleotide reductase (16). Attempts to stimulate pyridine nucleotide reduction with hydrogen by addition of this enzyme to extracts which are unable to carry out this reaction (preparations from cell lot B) or extracts which show a parabolic response to protein concentration (aged preparations from cell lot A) gave negative results.

The relationship between FAD and the cofactor in boiled cell extracts of *C. kluyveri* is now under investigation. The present data indicate that the cofactor has a higher affinity for the enzyme(s) and a higher rate of reactivity at saturation than FAD. These results suggest that FAD and the cofactor are not identical. The possibility exists, however, that there are two cofactors present in the boiled cell extract which are required for maximal activity and that one of these may be identical with FAD. A definitive answer is contingent upon the eventual isolation and characterization of the active component(s).

SUMMARY

The alternative routes of hydrogen oxidation by *Clostridium kluyveri* have been investigated with the following results.

1. Flavin nucleotide and dye reduction do not require the participation of a heat-stable cofactor which is necessary for pyridine nucleotide reduction.

2. Flavin adenine dinucleotide is partially effective in replacing the cofactor but is not identical with it.

3. At least two enzymes are involved in pyridine nucleotide reduction.

4. An enzyme not required for dye and flavin reduction is necessary for diphosphopyridine nucleotide reduction.

Acknowledgment—I should like to express my sincere appreciation to Dr. Earl R. Stadtman, who originally suggested this problem, for his generous and invaluable advice.

REFERENCES

1. Korkes, S., in W. D. McElroy and H. B. Glass (Editors), *Phosphorous metabolism, Vol. II*, Johns Hopkins Press, Baltimore, 1952, p. 502.
2. Korkes, S., *J. Biol. Chem.*, **216**, 737 (1955).
3. Peck, H. D., and Gest, H., *Biochim. et Biophys. Acta*, **15**, 587 (1954).
4. Packer, L., and Vishniac, W., *Bacteriol. Proc.*, 111 (1954).
5. Wittenberger, C. L., and Repaske, R., *Bacteriol. Proc.*, 106 (1958).
6. Kinsky, S. C., *Federation Proc.*, **17**, 254 (1958).
7. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1035 (1949).
8. Kornberg, A., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. I*, Academic Press, Inc., New York, 1955, p. 441.
9. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
11. Nason, A., and Evans, H. J., *J. Biol. Chem.*, **202**, 655, (1953).
12. Kornberg, A., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. II*, Academic Press, Inc., New York, 1955, p. 655.
13. Packer, L., and Vishniac, W., *Biochim. et Biophys. Acta*, **17**, 153 (1955).
14. Krasna, A., Riklis, E., and Rittenberg, D., *Federation Proc.*, **17**, 258 (1958).
15. Vishniac, W., and Ochoa, S., in W. D. McElroy and H. B. Glass (Editors), *Phosphorous metabolism, Vol. II*, Johns Hopkins Press, Baltimore, 1952, p. 467.
16. San Pietro, A., and Lang, H. M., *J. Biol. Chem.*, **231**, 211 (1958).

Pyridine Nucleotide Transhydrogenase

VII. DETERMINATION OF THE REACTIONS WITH COENZYME ANALOGUES IN MAMMALIAN TISSUES*

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Since our initial report (1) on the occurrence of pyridine nucleotide transhydrogenase activity in animal tissue mitochondria and the purification of such an enzyme from beef heart mitochondria, there have been a number of papers which have confirmed and extended our findings (2–5). In our first publication we reported that the animal tissue mitochondrial preparations catalyzed the following reversible reaction:

$$\text{TPNH} + \text{DPN} \rightleftharpoons \text{TPN} + \text{DPNH} \quad (1)$$

In addition, a second transfer reaction between the oxidized and reduced forms of DPN was described. This reaction was followed by means of DPN labeled with C^{14} in the nicotinamide moiety (6), or by a reaction involving deamino-DPN[1] (1) as illustrated in the following equation:

$$\text{DPNH} + \text{deamino-DPN} \rightarrow \text{DPN} + \text{deamino-DPNH} \quad (2)$$

During the course of studies with flavoproteins, it was noted that certain of these enzyme preparations catalyzed the oxidation of DPNH by the acetylpyridine analogue of DPN (7–9) according to Equation 3:

$$\text{DPNH} + \text{acetylpyridine (DPN)} \rightarrow \text{DPN} + \text{acetylpyridine (DPNH)} \quad (3)$$

This reaction could be followed directly in the spectrophotometer utilizing the difference in absorption maxima of DPNH and its reduced acetylpyridine analogue (8). DPNH and reduced deamino-DPN have identical spectra. Furthermore, Reaction 3 goes to completion because of the more positive potential of the acetylpyridine analogue of DPN (9).

Studies measuring transhydrogenase activity by cytochrome *c* reduction and oxygen uptake (10) indicated that the enzyme was present in rat liver mitochondria in higher concentrations than those obtained by our original assay system, which involved the generation of DPNH mediated by the transhydrogenase in the presence of the isocitric dehydrogenase system and catalytic amounts of TPN; the reaction was followed at 340 mμ. It appeared to us that the oxidation of TPNH by the acetylpyridine analogue of DPN, as given in the following equation

$$\text{TPNH} + \text{acetylpyridine (DPN)} \rightarrow \text{TPN} + \text{acetylpyridine (DPNH)} \quad (4)$$

could be a more sensitive and perhaps more meaningful method for measuring transhydrogenase activity.

It is the purpose of this paper to describe the application of the above reaction to the determination of transhydrogenase activity in animal tissues and also to give further information on reaction 3. Data will also be given on the following two reactions:

$$\text{DPNH} + \text{acetylpyridine (TPN)} \rightarrow \text{DPN} + \text{acetylpyridine (TPNH)} \quad (5)$$

$$\text{TPNH} + \text{acetylpyridine (TPN)} \rightarrow \text{TPN} + \text{acetylpyridine (TPNH)} \quad (6)$$

Information as to whether one or more enzymes are involved in the various transfer reactions of the pyridine nucleotides will also be presented.

EXPERIMENTAL

DPN, DPNH, and TPN were obtained from the Pabst Laboratories. TPNH was obtained from the Sigma Chemical Company. The acetylpyridine and pyridine-3-aldehyde analogues of DPN were obtained from the Pabst Laboratories. The acetylpyridine analogue of TPN was prepared by the transglycosidase reaction as described previously (8). The thionicotinamide analogue of DPN was prepared by Dr. B. M. Anderson (11). The digitonin used was a preparation of the Merck Chemical Company. L-Thyroxine and triiodothyronine were obtained from Smith, Kline and French Laboratories.

Preparation of Tissues—Mitochondria, microsomes, and soluble fractions were obtained from rat and rabbit tissues as described by Kielley and Kielley (12). The tissues were forced through a stainless steel press with holes about 1 mm. in diameter, and the weight of the tissues was estimated by displacement of 0.25 M sucrose in a graduated cylinder. Homogenization was carried out in a TenBroeck all-glass, hand homogenizer in a 10-fold dilution of 0.25 M sucrose. The particles sedimenting between 600 × *g* and 6,000 × *g*, and 25,000 × *g* and 105,000 × *g* were considered to be the mitochondrial and microsomal

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† Postdoctoral Fellow of the National Cancer Institute of the National Institutes of Health.

[1] The abbreviations used are: acetylpyridine (DPN) and acetylpyridine (DPNH), 3-acetylpyridine analogues of DPN and DPNH, respectively; acetylpyridine (TPN) and acetylpyridine (TPNH), 3-acetylpyridine analogues of TPN and TPNH respectively; deamino-DPN, hypoxanthine analogue of DPN.

fractions, respectively. Beef heart mitochondria were prepared as described previously (1), or by a modification of the method of Singer (13).

Concentration of Analogues—The concentration of the acetylpyridine analogue of DPN was measured by reduction with yeast alcohol dehydrogenase and ethanol with semicarbazide as a trapping reagent. The extinction coefficient of the reduced analogue at 365 mμ was taken as 7.8 (8).[2] The concentration of the acetylpyridine analogue of TPN was determined by forming the cyanide addition product (8).

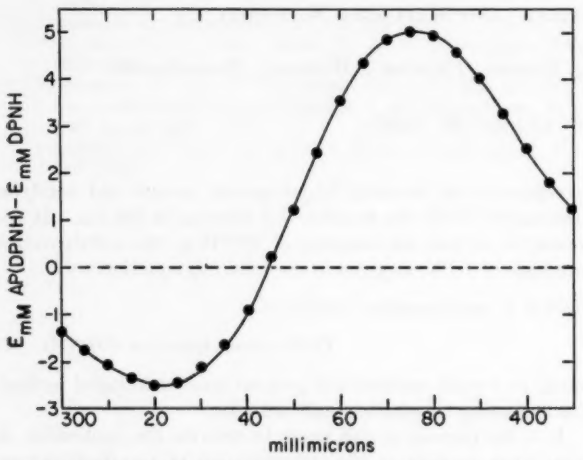

FIG. 1. Calculated difference of absorption between the acetylpyridine analogue of DPNH and DPNH. E_{mM} for DPNH is taken as 6.22 at 340 mμ, and for the analogue as 7.8[2] at 365 mμ, at the maximum of 375 mμ, E = 5.1.

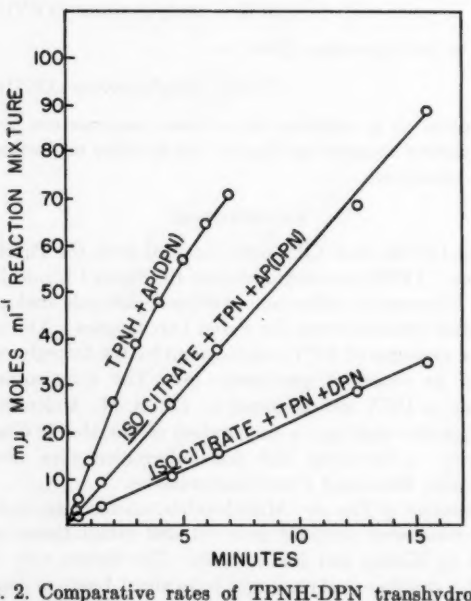

FIG. 2. Comparative rates of TPNH-DPN transhydrogenase determined with DPN and the acetylpyridine analogue of DPN (APDPN) as acceptors. The reaction mixtures are as described in the text, with the use of digitonized mitochondria from 25 mg. of rat liver, fresh weight. The reaction with TPNH is followed at pH 6.5. The reactions with isocitrate dehydrogenase and isocitrate (1) are followed at pH 7.5.

Estimation of Mitochondrial Protein—Of the mitochondrial suspensions, 0.5 to 1 ml. is precipitated with 5 ml. of 10 per cent trichloroacetic acid, extracted with hot 95 per cent ethanol and ether, and air dried. The residues are dispersed in 0.5 to 1 ml. of 0.5 N NaOH overnight. Aliquots of 0.1 ml. of the resulting solutions, diluted with 1.5 volumes of water, are assayed by the procedure of Lowry (14), with bovine serum albumin as a standard. By this method, approximately 20 mg. of mitochondrial protein are equivalent to a gm. of fresh rat liver.

RESULTS

Reaction Measurement—The reaction of TPNH and DPNH with pyridine nucleotide analogues was followed at room temperature in a 3-ml. cuvette with a 1-cm. light path in a Beckman model DU spectrophotometer. The contents of the cuvette were 0.3 mmole of potassium phosphate buffer, pH 6.5 or 7.5; 3 μmoles of KCN; 0.4 μmole of reduced pyridine nucleotide; and 0.6 μmole of acetylpyridine analogue of DPN in a final volume of 3 ml. The rate of reaction was estimated with the spectral difference of DPNH and its acetylpyridine analogue shown in Fig. 1. Generally the reaction is followed at 375 mμ, where the spectral difference is 0.0051 optical density unit per mμmole of analogue reduced. Small corrections for endogenous oxidation of reduced pyridine nucleotides were applied, when necessary, by subtracting the values obtained from a control cuvette without analogue acceptor. The rates of endogenous oxidation of reduced pyridine nucleotides in whole mitochondrial preparations are generally quite small, amounting to no more than 5 to 10 per cent of the optical density change at 375 mμ. The reduced analogues under the conditions of the experiments are oxidized at a slow rate; no corrections were made for this oxidation during the course of the transhydrogenase reactions, and, therefore, the rates reported represent minimal values. A study of the rate of reaction followed at several wave lengths indicated no significant effect of the pyridine nucleotide analogues on the endogenous oxidation of TPNH or DPNH in mitochondrial preparations.

Efficiency of Acetylpyridine Analogues—The rates of reaction of the TPNH-DPN transhydrogenase reaction with the use of DPN and its acetylpyridine analogue as acceptors are compared in Fig. 2. Under the conditions studied, the reaction is 5 to 6 times faster with the analogue as acceptor than with DPN.

Effect of Digitonin on Rate of Reaction—Digitonin was used originally (1) to extract transhydrogenase activity in a nonsedimentable form from beef heart particles. Treatment of mitochondria with digitonin before assay has been adopted for routine estimation of rates of reactions in mitochondria. Table I shows the effect of digitonin concentration on the activities of Reactions 4 and 3. In a typical preparation, mitochondria are prepared in sucrose to a final concentration representing 0.2 to 0.25 ml. of tissue press per ml. of suspension. Before assay, 1 ml. of mitochondrial suspension is mixed with 1 ml. of 1 per cent digitonin adjusted to about pH 7.5 (0.02 m.eq. of NaOH per 10 ml. of solution). Routinely, 0.05 ml. of mixture is assayed 10

[2] Recent data from the Pabst Laboratories with the use of a purer preparation of the reduced acetylpyridine analogue of DPN indicate the mM extinction coefficient to be 9.1 rather than the 7.8 value originally reported.

TABLE I

Activation by digitonin of rat liver mitochondrial transhydrogenases

Digitonin concentration	TPNH + acetylpyridine (DPN)	DPNH + acetylpyridine (DPN)
	Analogue reduced per min. per ml. of reaction mixture*	
%	*mμmoles*	*mμmoles*
0	4.65	8.24
0.125	7.57	8.12
0.25	7.78	11.7
0.50	8.47	14.0
1.0	6.92	13.9

* Mitochondria equivalent to 10 mg. of fresh liver were used. A mitochondrial suspension of 20 mg. per ml., fresh weight equivalent, was diluted with equal volumes of digitonin to yield the indicated concentrations.

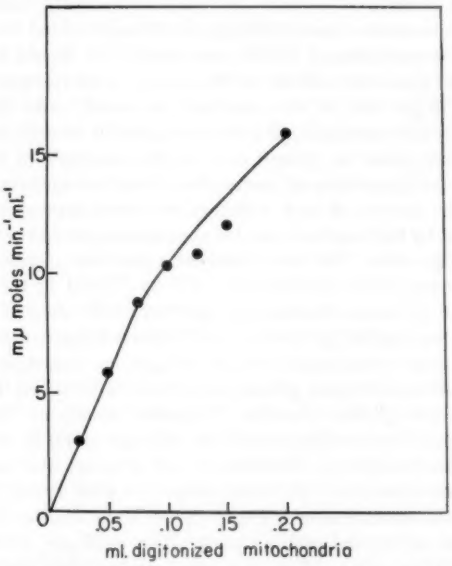

FIG. 3. The effect of concentration of digitonized rat liver mitochondria on the TPNH-acetylpyridine (DPN) transhydrogenase. One volume of mitochondrial suspension is added to one volume of 1 per cent digitonin to yield the equivalent of 10 mg. of fresh tissue per 0.10 ml. mixture. The rates are expressed in mμmoles per ml. of reaction mixture. The reaction is followed at 365 mμ. Conditions are described in the text. The reaction is carried out at pH 6.5 at 25°.

to 20 minutes after addition of digitonin, but no loss of activity was observed after several hours of storage of the digitonized suspension in an ice bath. Vignais and Vignais (4) have reported similar effects of digitonin on mitochondrial reaction rates. The importance of the use of small volumes of digitonized mitochondria for quantitative assay is shown in Fig. 3, where a loss of proportionality between activity and enzyme concentration is obtained with increasing amounts of enzyme. Under the conditions described above, the mitochondrial fraction from 1 gm. of fresh rat liver catalyzes at pH 6.5 the reduction of 3.5 to 4 μmoles of the acetylpyridine analogue of DPN by TPNH per minute.

Pigeon liver mitochondria show little transhydrogenase activity when assayed directly by the reduction of the acetylpyridine analogue of DPN when either TPNH or DPNH are donors. On treatment with digitonin there is a remarkable increase in both activities (see Fig. 4) and the rates are comparable to those found in rat liver. It is evident from these observations that precautions are essential in interpreting the relative concentrations of transhydrogenase activities in mitochondria.

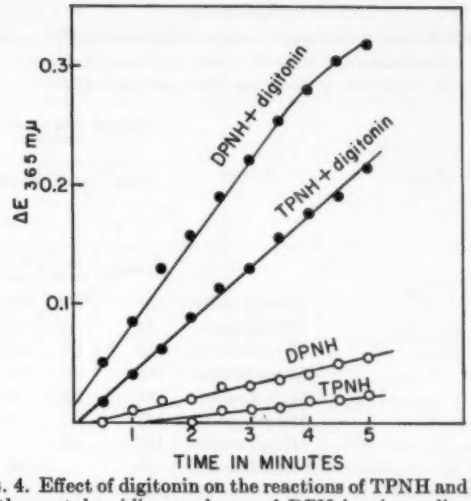

FIG. 4. Effect of digitonin on the reactions of TPNH and DPNH with the acetylpyridine analogue of DPN in pigeon liver mitochondria. Mitochondria from 18 mg. of liver, fresh weight. O——O, mitochondria preincubated 10 minutes in the reaction mixture; ●——●, mitochondria lysed in 0.5 per cent digitonin. Conditions are described in the text. The reaction is carried out at pH 6.5 at 25°.

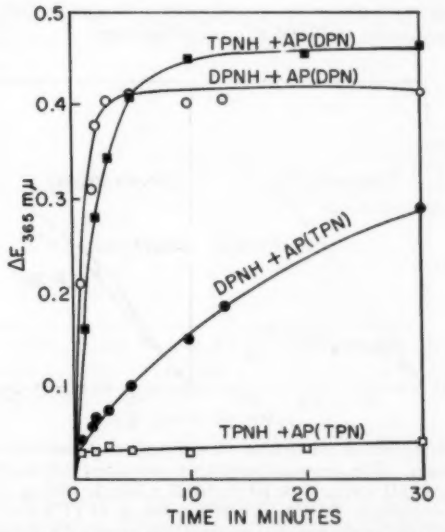

FIG. 5. Rates of various transhydrogenases in digitonin extracts (1) of beef heart mitochondria. APDPN represents the acetylpyridine analogue of DPN; APTPN, the acetylpyridine analogue of TPN. The conditions are described in the text.

Occurrence of Reactions—Relative rates of reduction of the acetylpyridine analogues of DPN and TPN by reduced pyridine nucleotides are shown in Fig. 5 for a digitonin extract (1) of beef

TABLE II

Distribution of transhydrogenase reactions in digitonized mitochondria of rat and rabbit tissues as measured by reduction of acetylpyridine analogue of DPN

Tissue	Fraction	Analogue reduced/min./mg. protein			
		TPNH	DPNH	TPNH	DPNH
		Rat		Rabbit	
		mμmoles	*mμ-moles*	*mμ-moles*	*mμ-moles*
Liver	Mitochondria	181	254	102	76
Liver	Microsomes	14	16		
Heart	Mitochondria	367	490	115	361
Kidney	Mitochondria	168	243	166	193
Brain	Mitochondria	34	290	25	89
Lung	Mitochondria	143	176	93	81
Testis	Mitochondria	112	234		
Spleen	Mitochondria			102	115
Skeletal Muscle	Mitochondria	137	290	*	99
Adrenal	Mitochondria			*	372
Novikoff Hepatoma	Mitochondria	27	59		
Placenta†	Mitochondria	32			
Placenta	Supernatant‡	0.8*			

* Negligible activity.

† Prepartum placentae, average about 10 mm. in diameter.

‡ Supernatant of 25,000 × *g* centrifugation.

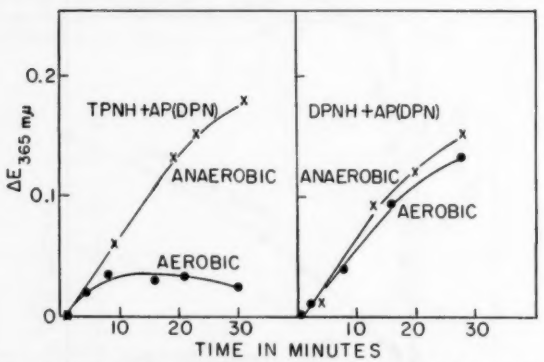

FIG. 6. Effect of anaerobiosis on Reactions 4 and 3 in rat liver microsomes. The reaction mixtures contained 0.1 M potassium phosphate, pH 6.8; 0.001 M KCN; 0.003 M $MgCl_2$; 600 μg. of acetylpyridine analogue of DPN (APDPN); 400 μg. of TPNH or DPNH; and 0.1 cc. of the microsomal preparation in a final volume of 3 cc. The reaction is started by the addition of the microsomes. The anaerobic experiments were carried out in a Thunberg-type cuvette.

heart mitochondria. Digitonin extracts of rat liver mitochondria show a similar spectrum of activities. As in the case of the beef heart mitochondria, the rat liver mitochondrial extracts fail to catalyze Reaction 6 and catalyze Reaction 5 at a considerably slower rate than Reaction 4. In the purification of the beef heart enzymes (1), the activity of the TPNH-DPN reaction follows closely that of the corresponding analogue reaction. The reaction of TPNH with the acetylpyridine analogue of TPN (Reaction 6) is virtually absent from all mitochondrial preparations tested. However, this reaction has been detected in appreciable amounts in the microsomal fraction of rat liver. It has also been found to be catalyzed by a diaphorase from chloroplasts and by a beef heart TPNH-cytchrome *c* reductase preparation by Weber and Kaplan (15), and lately by us in the soluble fraction of Chromatium sonicates. The low rate of reaction of DPNH with the acetylpyridine analogue of TPN is in accord with the relatively slow rate of the reverse transhydrogenase reaction (Reaction 1), previously observed in animal tissue mitochondria (1). The distribution of the major transhydrogenase reactions in rat and rabbit tissue fractions is shown in Table II. These results are in general agreement with previous data (1). The greater sensitivity of the present method allows more reliable estimates of rates in tissues with low activities. It appears, at least in the case of liver, that some of the older data may have been complicated by the impermeability of the mitochondria. Rat liver microsomes display much less transhydrogenase activity than do mitochondria. Fig. 6 shows the effect of anaerobic conditions on Reactions 4 and 3 in microsomes. The failure to observe the reaction of the acetylpyridine analogue of DPN with TPNH under aerobic conditions may be due to the fact that at the concentration used (0.001 M), the cyanide added fails to inhibit the oxidation of TPNH completely. It should be noted that the anaerobic activity of Reaction 4 in microsomes is only about 25 per cent of that obtained aerobically with digitonin. As previously noted (1), all pyridine nucleotide transfer reactions are either absent or present only in trace amounts in the 105,000 × *g* supernatants of mammalian tissue homogenates. Further, the amount of such activities recovered appears to be influenced by the length of time the samples are exposed to the high centrifugal field. We have detected a low rate of reduction of the acetylpyridine analogue of DPN by TPNH in the soluble fraction of human placenta in agreement with some of the observations reported by Talalay and Williams-Ashman (16). The results were complicated by an endogenous reduction of the analogue several times greater than found with added TPNH.

Reduction of Other Pyridine Nucleotide Analogues—We have found that the pyridine-3-aldehyde analogue of DPN (8, 9) can act as an acceptor in Reactions 3 and 4 in rat liver and beef heart mitochondria. We have studied in some detail the role of the thionicotinamide analogue of DPN[3] as acceptor in these reactions in rat and rabbit tissues. This analogue (17) is only about 50 per cent as effective as the acetylpyridine analogue as an acceptor in Reaction 4. On the other hand, it is as active or more active than the acetylpyridine analogue in Reaction 3, in spite of its lower oxidation-reduction potential (17). This was particularly marked with the partially purified enzyme from rat liver mitochondria (18); here the activity with the thionicotinamide analogue was found to be 4 to 5 times greater than with the acetylpyridine analogue. These studies will be reported in detail elsewhere.

Affinity Constants—The Michaelis constants of the various pyridine nucleotides in Reactions 4 and 5 have been determined. K_m values for Reaction 4 are 7.5×10^{-5} M for TPNH and 1.5×10^{-5} M for the acetylpyridine analogue of DPN as determined by Lineweaver-Burk plots. Similarly, in Reaction 5, the values are 9.3×10^{-6} M and 7.7×10^{-5} M for DPNH and the acetylpyridine analogue of TPN, respectively. The latter determina-

[3] The reduced thionicotinamide analogue of DPN has an absorption maximum at 400 mμ and the reaction can be followed readily by the difference between the reduced spectrum of this compound and that of DPNH. The pig heart diaphorase transfer reaction has been studied in this manner (17).

TABLE III

*Effect of storage on rat liver mitochondrial transhydrogenases**

Days stored at 4°	DPNH + acetylpyridine (DPN)	TPNH + acetylpyridine (DPN)
	$\Delta A_{365\,m\mu}$ from 2 to 7 min.	
0	0.243	0.118
1	0.230	0.082
4	0.213	0.060
5	0.205	0.025
6	0.215	0.033
11	0.227	0.020

* The preparation used was a calcium phosphate gel supernatant of a digitonin extract of rat liver mitochondria.

TABLE IV

Effect of thyroxine and triiodothyronine on beef heart transhydrogenases

Suspension of beef heart mitochondria added to cuvette at zero time; acetylpyridine analogue of DPN used in all reactions.

Reduced pyridine nucleotide	Inhibitor	Concentration	mμmoles $min.^{-1}$ $ml.^{-1}$ of reaction mixture
		M	
TPNH	None		8.09
TPNH	Thyroxine	3.7×10^{-5}	5.37
TPNH	Triiodothyronine	3.7×10^{-5}	2.84
DPNH	None		19.9
DPNH	Triiodothyronine	3.7×10^{-5}	19.9

tions are in general agreement with the constants reported by Humphrey (2) for the reaction between DPNH and TPN.

Identity of Reactions—Differentiation of the transhydrogenase reactions of animal tissue mitochondria has been obtained from several types of evidence. Table III illustrates that the system responsible for the catalysis of Reaction 3 is stable on storage at 4°, whereas the transfer between TPNH and the acetylpyridine analogue of DPN (Reaction 4) deteriorates. Sonic oscillation of rat liver and beef heart mitochondria leads to a considerable extraction of the activity represented by Reaction 3 in the 105,000 × *g* supernatant fluid, but fails to extract the activity represented by Reaction 4 in significant amounts. Reaction 4 was found to be far more sensitive to treatment with solvents and to dialysis than Reaction 3. We have been able to substantiate the report of Ball and Cooper (3) that thyroxine inhibits the TPNH-DPN transhydrogenase activity when assayed by Reaction 4. However, triiodothyronine, and in separate experiments, thyroxine, had no effect on the reaction of DPNH with the acetylpyridine analogue of DPN (Table IV). Fig. 7 shows the effect of triiodothyronine on the forward and reverse reaction of transhydrogenase (Reaction 4). Essentially identical inhibitions were obtained by triiodothyronine in the two reactions. We have further shown that 2,4-dinitrophenol has little or no effect on the TPNH reaction with the acetylpyridine analogue of DPN (4 per cent inhibition at 10^{-4} M).

The transhydrogenase reaction between TPNH and DPN does not appear to be associated with the diaphorase activities of mitochondria (19, 18), since the latter are readily extractable by

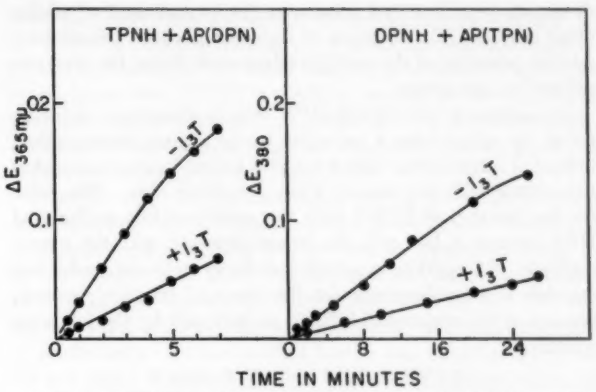

FIG. 7. The effect of triiodothyronine on the TPNH + acetylpyridine analogue of DPN (APDPN), Reaction 4, and DPNH + acetylpyridine analogue of TPN (APTPN), Reaction 5, reactions in a solubilized (1) preparation of beef heart mitochondria. Enzyme, 0.2 ml., was used in Reaction 4 and 0.3 ml. was used in Reaction 5. I_3T refers to 1×10^{-4} M triiodothyronine. The reactions were run at pH 6.5. The conditions are described in the text.

TABLE V

Effect of heating on TPNH-acetylpyridine (DPN) transhydrogenase and TPNH diaphorase

Suspension of beef heart mitochondria in 0.05 M potassium phosphate buffer, pH 7.0, was heated at 56° for various time intervals.

	Acceptor	
	2,6-dichlorophenolindophenol*	Acetyl pyridine analogue of DPN
min.	*mμmoles reduced $min.^{-1}$ $ml.^{-1}$ of reaction mixture*	
0	3.3	32
1	2.6	25
2	3.5	15
4	3.2	0

* Reaction mixture buffered with 0.3 ml. of tris(hydroxymethyl) aminomethane buffer, 0.2 M, pH 7.6. Diaphorase activity was assayed as described previously (18).

sonic oscillation in contrast to the former. Further, it appears that TPNH diaphorase is quite resistant to heating, whereas the transhydrogenase which catalyzes the interaction between the two forms of the pyridine coenzymes is readily destroyed (Table V). We have failed to obtain any evidence for Reaction 4 either in reaction mixtures containing DPNH or TPNH specific diaphorases, or the nonspecific diaphorase A of rat liver mitochondria (18). This latter enzyme reacts with both TPNH and DPNH.

DISCUSSION

The data presented in this paper illustrate the advantage of the use of pyridine coenzyme analogues in measuring the pyridine nucleotide transhydrogenase reactions. The higher values obtained with the analogues may be related, in part, to the high oxidation-reduction potential of the acetylpyridine coenzymes (19). Furthermore, the difference in absorption maxima and the higher extinction coefficients of the reduced analogues makes possible a simple and direct method for following the transhy-

drogenase reactions and eliminates the requirement of adding other enzymes (1). Because of the more positive oxidation-reduction potential of the acetylpyridine nucleotides, the reactions proceed to completion.

Measurement of TPNH-DPN transhydrogenase activity, either by cytochrome *c* reduction or by oxygen consumption, indicated considerably higher activity for rat liver mitochondria than found with the original assay procedure (10). The value for the reaction of TPNH with the acetylpyridine analogue of DPN is more in line with the results obtained with the former methods. It might be expected that the cytochrome *c* reduction reaction is more favorable for the assay of transhydrogenase, because of the removal of DPNH, as indicated by the following equations:

$$\text{TPNH} + \text{DPN} \rightleftharpoons \text{TPN} + \text{DPNH}$$

$$\text{DPNH} + \text{cytochrome } c\ \text{Fe}^{+++} \rightarrow \text{DPN} + \text{cytochrome } c\ \text{Fe}^{++}$$

Similar considerations would hold for the removal of DPNH by oxygen uptake. With the use of the acetylpyridine analogue of DPN as acceptor, the equilibrium problems are avoided and the reaction proceeds much more rapidly.

In discussing the equilibrium of the transhydrogenase reaction, it should be pointed out that under normal conditions in mitochondria the DPN is probably bound to protein. As indicated by Theorell and Bonnichsen (20) the binding of DPN increases the oxidation-reduction potential of the DPNH-DPN couple some 0.06 volt. This, therefore, would favor energetically the reduction of DPN in the transhydrogenase reaction as occurring under natural conditions.

It is difficult to attempt to account for the respiratory rates in terms of measurement of individual enzymatic steps. This is pointed out by Ernster and Navazio (21) in reference to a role played in respiration by a possible DPN-linked isocitrate dehydrogenase of rat liver mitochondria. These authors, on the basis of low values obtained with their transhydrogenase assays on rat liver mitochondria, have ruled out transhydrogenase (Reaction 1) as a factor in isocitrate oxidation by mitochondria in favor of the aforementioned DPN-linked isocitrate dehydrogenase. With our present method of assay, we have obtained values for the rate of TPNH-DPN transhydrogenase 40 to 50 times greater than those reported by Ernster and Navazio (21), sufficient to accommodate the rate of reduction of TPN by isocitrate and about 5 times greater than the isocitrate-coupled oxygen uptake observed by these authors. We have observed the rate of the transhydrogenase reaction to be in the same order of magnitude as TPN-isocitrate dehydrogenase and DPNH-cytochrome *c* reductase in rat liver mitochondria.

Although the acetylpyridine analogues of DPN and TPN are active with the animal tissue dehydrogenases, they will not act as acceptors in the *Pseudomonas fluorescens* transhydrogenase system (15). Hence, caution must be used when applying the analogue method for the assay of transhydrogenase activities of different sources. As noted above, DPNH will react with the thionicotinamide analogue of DPN. The relative rates of reaction with the two analogues appear to be different in different tissues. This suggests some structural differences between the enzymes of different tissues catalyzing Reaction 3.

The Pseudomonas enzyme (22) catalyzes the exchange between TPNH and DPN as well as the DPNH-DPN reaction. These two activities have not been separated, and it appears possible that they are due to one protein. The experiments reported in this paper indicate that these reactions are catalyzed by two different proteins in the animal tissue mitochondria. The reaction of DPNH with the acetylpyridine analogue of DPN has been solubilized by sonic oscillation and under these conditions the reaction of TPNH with the acetylpyridine analogue of DPN remains in the insoluble fraction. Although the reaction of DPNH with the acetylpyridine analogue of DPN appears to be associated with diaphorase activity, the enzyme appears to be distinct from the nonspecific diaphorase which is also present in mitochondria (18).

It is of interest that the reaction of DPNH with the acetylpyridine analogue of TPN proceeds at a much slower rate than the converse reaction of TPNH with the acetylpyridine analogue of DPN. This difference in rate has also been observed with the natural coenzymes (1). It thus appears that the animal tissue transhydrogenase has some properties in common with the bacterial system, since with the latter enzyme the DPNH-TPN reaction is very sluggish and will not proceed unless 2'-adenylic acid is added (23). In the rat liver TPNH-DPN transhydrogenase, the affinities of the reduced pyridine nucleotides and pyridine nucleotide analogue acceptors are such as to possibly afford an explanation for the difference in rates for the forward and reverse reactions. It will be noted that the preferred direction for Reaction 1 is consistent with Reaction 4 being more active than Reaction 5 and is in the direction of the nucleotides with the lowest K_m value. The difference in rate of the forward and reverse reaction may be of significance as a physiological regulating mechanism of electron transfer in mitochondria.

Talalay and Williams-Ashman (16) have reported that the estradiol-β-17 dehydrogenase from human placenta can act as a transhydrogenase. This dehydrogenase is both TPN and DPN linked and Talalay and Williams-Ashman have suggested that the net transfer of hydrogen or electrons from TPNH to DPN mediated by placental extracts is effected by alternate oxidation and reduction of catalytic amounts of estradiol-β-17. It is suggested by these authors that transhydrogenase may be such an enzyme. It should be emphasized that the steroid dehydrogenase activity is located in the soluble fraction of the cytoplasm whereas essentially all of the transhydrogenase activity which we have detected is present in particulate fractions of the cell. It is of interest that the properties of rat liver mitochondrial transhydrogenase are different from those reported by Talalay *et al.* (24) for his placental enzyme, both with respect to affinity of pyridine nucleotides for the enzyme and the relative rates with pyridine nucleotide analogues. The possibility exists that the TPNH-DPN transhydrogenase of the mitochondria may be due to a TPN-DPN-linked dehydrogenase which conceivably may involve steroids. However, this has yet to be demonstrated, and until this point has been clarified, confusion might arise if the activity described by Talalay and Williams-Ashman is linked with the transhydrogenase system which we have studied in mitochondria. Further work on the purification of mitochondrial transhydrogenases is now under way.

Navazio *et al.* (25) have attempted to account for the oxidation of TPNH in rat liver by the lactic dehydrogenase activity of the soluble fraction of rat liver homogenates. This attempt becomes necessary if one ignores the potent TPN-DPN transhydrogenase we have demonstrated in mitochondria of rat liver and other tissues, and assumes a DPN-linked isocitrate dehydrogenase in mitochondria to account for isocitrate oxidation. In spite of many attempts, we have failed to demonstrate any

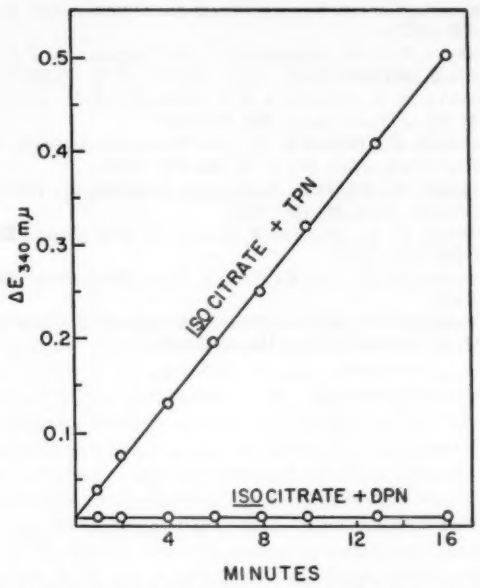

FIG. 8. Rate of isocitrate-linked TPN and DPN reduction in depleted rat liver mitochondria (25). Mitochondria from 6.4 mg. of rat liver, fresh weight, were used. The reaction was carried out at pH 7.5, in potassium phosphate buffer, as described in the text, with 0.5 μmole of either DPN or TPN. The reaction was started by addition of pyridine nucleotide.

trace of DPN reduction with isocitrate in rat liver mitochondria where the pre-existing pyridine nucleotides have been removed by proper depletion (26). The results of such an experiment are shown in Fig. 8. The concept that the soluble lactic dehydrogenase of rat liver will account for TPNH oxidation of the rat liver cell can be criticized on several counts. It obviously cannot account for isocitrate oxidation in mitochondrial preparations, which must be assumed to be associated with TPN in the absence of convincing evidence for a mitochondrial DPN-linked isocitrate dehydrogenase in rat liver. It is doubtful that this process could account for a net TPNH oxidation since DPNH formed stoichiometrically with pyruvate during glycolysis would be preferentially oxidized by the pyruvate. Navazio *et al.* (25) have reported that the oxidation of TPNH by pyruvate with lactic dehydrogenase is about 2.5 per cent that of DPNH. Furthermore, the presence of high concentrations of DPN in the rat liver soluble fraction (27) would materially decrease this reaction, according to the data of Navazio *et al.* (25) on the inhibition of TPNH oxidation by pyruvate in the presence of DPN. As indicated previously (28), lactic dehydrogenase does not carry out a transfer of reduction state from TPNH to DPN. We have confirmed this with the use of the acetylpyridine analogue of DPN in the presence of catalytic or substrate concentrations of pyruvate. It should be noted that a net transfer of hydrogen or electrons from TPNH to DPN is implicit in the suggestion that lactic dehydrogenase can account for TPNH oxidation, unless it is further suggested that an alternate pathway exists for lactate metabolism, not involving DPN.

In confirmation of Ball and Cooper's work (3), we have found that thyroxine and triiodothyronine, as well, will inhibit the transhydrogenase reaction as measured by Reactions 4 and 5. It is of interest that these compounds do not affect the reaction of DPNH with the acetylpyridine analogue of DPN. The significance of this inhibition is as yet not clear since we have found in preliminary experiments that hyper- and hypothyroid states do not produce notable changes in transhydrogenase levels of rat liver mitochondria.

SUMMARY

1. A method is described for the assay of transhydrogenase activity through the use of the acetylpyridine analogues of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). This method, which involves the transfer of hydrogens or electrons from reduced triphosphopyridine nucleotide (TPNH) to the acetylpyridine analogue of DPN, gives values considerably higher than those obtained by other methods. It has also been found, in confirmation of earlier work, that the highest levels of transhydrogenase are located in mitochondria of heart, kidney, and liver. Relatively little activity is found in brain. Nearly all of the transhydrogenase activity is located in the mitochondria, although some evidence has been obtained for the presence of low levels of transhydrogenase activity in rat liver microsomes.

2. It has been found that the rat liver and beef heart transhydrogenases promote the reaction between TPNH and the acetylpyridine analogue of DPN at a much faster rate than the analogous reverse reaction, the reaction of reduced diphosphopyridine nucleotide (DPNH) with the acetylpyridine analogue of TPN.

3. Mitochondrial preparations will also promote a reaction between DPNH and the acetylpyridine analogue of DPN. Evidence is presented which indicates that this reaction is catalyzed by a different enzyme than that which is responsible for the reaction of TPNH with the acetylpyridine analogue of DPN.

4. Thyroxine and triiodothyronine have been found to inhibit the reaction of TPNH and the acetylpyridine analogue of DPN, whereas these compounds do not influence the reaction of DPNH with the acetylpyridine analogue of DPN.

5. The significance of the results of the acetylpyridine analogue assay for transhydrogenase reactions in whole mitochondrial preparations is discussed. A discussion of the various transhydrogenase reactions and their possible nature is presented.

REFERENCES

1. KAPLAN, N. O., COLOWICK, S. P., AND NEUFELD, E. F., *J. Biol. Chem.*, **205**, 1 (1953).
2. HUMPHREY, G. F., *Biochem. J.*, **65**, 546 (1957).
3. BALL, E. G., AND COOPER, O., *Proc. Natl. Acad. Sci. U. S.*, **43**, 357 (1957).
4. VIGNAIS, P. V., AND VIGNAIS, P. M., *J. Biol. Chem.*, **229**, 265 (1957).
5. KIELLEY, W. W., AND BRONK, J. R., *J. Biol. Chem.*, **230**, 521 (1958).
6. KAPLAN, N. O., COLOWICK, S. P., ZATMAN, L. J., AND CIOTTI, M. M., *J. Biol. Chem.*, **205**, 31 (1953).
7. KAPLAN, N. O., AND CIOTTI, M. M., *J. Am. Chem. Soc.*, **76**, 1713 (1954).
8. KAPLAN, N. O., AND CIOTTI, M. M., *J. Biol. Chem.*, **221**, 823 (1956).
9. KAPLAN, N. O., CIOTTI, M. M., AND STOLZENBACH, F. E., *J. Biol. Chem.*, **221**, 833 (1956).
10. KAPLAN, N. O., SWARTZ, M. N., FRECH, M. E., AND CIOTTI, M. M., *Proc. Natl. Acad. Sci. U. S.*, **42**, 481 (1956).
11. ANDERSON, B. M., CIOTTI, C. J., AND KAPLAN, N. O., *J. Biol. Chem.*, in press.

12. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191**, 485 (1951).
13. Singer, T. P., Kearney, E. B., and Bernath, P., *J. Biol. Chem.*, **223**, 599 (1956).
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
15. Weber, M. M., and Kaplan, N. O., *J. Biol. Chem.*, **225**, 909 (1957).
16. Talalay, P., and Williams-Ashman, H. G., *Proc. Natl. Acad. Sci. U. S.*, **44**, 15 (1958).
17. Anderson, B. M., and Kaplan, N. O., *J. Biol. Chem.*, in press.
18. Stein, A. M., and Kaplan, N. O., *Biochim. et Biophys. Acta*, **29**, 452 (1958).
19. Ernster, L., *Federation Proc.*, **17**, 216 (1958).
20. Theorell, H., and Bonnichsen, R. K., *Acta Chem. Scand.*, **5**, 1105 (1951).
21. Ernster, L., and Navazio, F., *Biochim. et Biophys. Acta*, **26**, 408 (1957).
22. Kaplan, N. O., Colowick, S. P., and Neufeld, E. F., *J. Biol. Chem.*, **195**, 107 (1952).
23. Kaplan, N. O., Colowick, S. P., Neufeld, E. F., and Ciotti, M. M., *J. Biol. Chem.*, **205**, 17 (1953).
24. Talalay, P., Hurlock, B., and Williams-Ashman, H. G., *Proc. Natl. Acad. Sci. U. S.*, **44**, 862 (1958).
25. Navazio, F., Ernster, B. B., and Ernster, L., *Biochim. et Biophys. Acta*, **26**, 416 (1957).
26. Hunter, F. E., Jr., and Ford, L., *J. Biol. Chem.*, **216**, 357 (1955).
27. Jacobson, K. B., and Kaplan, N. O., *J. Biol. Chem.*, **226**, 603 (1957).
28. Colowick, S. P., Kaplan, N. O., Neufeld, E. R., and Ciotti, M. M., *J. Biol. Chem.*, **195**, 95 (1952).

Some Effects of Phlorizin on the Metabolism of Mitochondria*

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The exact nature of the phlorizin inhibition of biological transport remains unknown. A number of different studies have shown effects of the glycoside on both membrane permeability (1, 2) and cellular metabolism (3, 4). Shapiro (3) found in 1947 that phlorizin in concentrations of 2×10^{-4} to 10^{-3} M reduces the aerobic utilization of glucose, pyruvate, and citrate and depresses creatine phosphorylation in a rat kidney mince. These observations were extended by Lotspeich and Keller (4) to homogenates of guinea pig kidney. They demonstrated in this preparation that phlorizin in similar concentrations inhibits the oxidation of all the substrates of the tricarboxylic acid cycle and that this inhibition can be completely prevented by an excess of adenine nucleotides.

Since the mitochondria contain the enzymes of the tricarboxylic acid cycle and associated oxidative phosphorylation, it seemed worthwhile to extend our studies to these subcellular structures. Accordingly the present paper presents experiments with isolated respiring mitochondria from both kidney and liver. In these organelles phlorizin produces a similar defect of oxidative metabolism which has a special relation to the steady state of the respiratory electron carrier system. Besides reporting these studies, this paper also serves to introduce the following one (5) on the effect of phlorizin on the swelling of mitochondria in isotonic sucrose. These experiments, we believe, largely explain the phlorizin defect in oxidative metabolism and also harmonize the disparate observations implicating phlorizin in both oxidative metabolism and membrane permeability.

EXPERIMENTAL

Studies on oxidative phosphorylation were carried out essentially as described by Copenhaver and Lardy (6). To the main compartment of the Warburg flasks the various components were added from isotonic solutions to give the following final concentrations in a 3-ml. volume: potassium phosphate buffer, pH 7.3, 13.3 mM; substrate (succinate, L-malate, β-hydroxybutyrate, or α-ketoglutarate), 6.7 mM; ATP, 2 mM; $MgSO_4$, 7.5 mM; cytochrome *c*, 0.012 mM; 0.5 ml. of mitochondria from 0.5 gm. of tissue; 0.25 M sucrose to a volume of 2.7 ml. When disodium malonate was used to limit the oxidation of α-ketoglutarate to one step (6), its concentration was 20 mM. Phlorizin, when used, was placed in the main compartment unless otherwise noted. The hexokinase preparation, 0.2 ml., and glucose, 0.1 ml. (for a final concentration of 16.7 mM) were added to the side arm. Saturated KOH, 0.2 ml., and a filter paper strip were placed in the center well. Triplicate flasks were incubated at 30° with air as the gas phase. After 3 minutes the side arm contents were tipped into the main compartment, and at 5 minutes the initial manometer readings were taken for oxygen uptake over the next 8 to 20 minutes. Deproteinization with 5 per cent trichloroacetic acid was carried out on one flask of each of triplicates at 5 minutes and on the respiration flasks immediately after the final manometer reading. Inorganic phosphate was determined by the method of Lowry and Lopez (7), and phosphate uptake was calculated as the difference between that remaining after respiration and that present in the flask deproteinized at 5 minutes. The P:O ratio was calculated as the ratio of the micromoles of inorganic phosphate taken up to the microatoms of oxygen used.

Mitochondria were prepared from rabbit kidney cortex (or other tissue when specified) in 0.25 M sucrose according to the method of Schneider (8). Each milliliter of final suspension represented 1 gm. of original tissue. Crude hexokinase was prepared by a method of Loomis (unpublished)[1] and contained about 300 Kunitz-McDonald units per ml. (9).

Cytochrome *c* and the sodium salts of ATP and ADP were obtained from the Sigma Chemical Company. Crystalline phlorizin was obtained from Bios Laboratories, Inc. This material was strongly glycosuric, showed a single spot with the R_f of phlorizin on the paper chromatogram (10), and showed the characteristic ultraviolet absorption peak of phlorizin at 285 mμ in the Beckman spectrophotometer.

RESULTS AND DISCUSSION

Studies on Efficiency of Oxidative Phosphorylation—An initial series of experiments, summarized in Table I, was done to test for a phlorizin effect on oxygen uptake and P:O ratio. Mitochondria from three tissues (rat liver, guinea pig kidney cortex, rabbit kidney cortex) were tested, and four different substrates were used (succinate, L-malate, β-hydroxybutyrate, and α-ketoglutarate with malonate). In every instance where phlorizin was present at 5×10^{-4} M or higher, oxygen utilization was inhibited and the efficiency of phosphorylation diminished. These results agree well with the report of Nagai (11) that mitochondria prepared from kidneys of rats given prolonged treatment with phlorizin showed lowered P:O ratios.

With these findings established, it was important to test the effect of phlorizin on the isolated hexokinase reaction, because an inhibition of phosphate transfer from ATP to glucose might conceivably account for the observed oxidative effects. This was done by using the Colowick and Kalckar (12) technique for

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[1] The method was kindly supplied to the authors by Dr. Roger L. Greif, Department of Physiology, Cornell University Medical College.

TABLE I

Effect of phlorizin on oxidation and phosphorylation in mitochondria from kidney and liver

Experiment No.	Substrate	Phlorizin concentration	Oxygen Utilization		P:O ratio	
			Control	Phlorizin	Control	Phlorizin
		M	μatoms/min./flask			
1*	α-Ketoglutarate + malonate	5×10^{-4}	.526	.320	3.0	0.5
2†	α-Ketoglutarate + malonate	5×10^{-4}	.380	.140	2.4	2.2
3	α-Ketoglutarate + malonate	5×10^{-4}	.473	.260	2.7	1.8
4	α-Ketoglutarate + mlaonate	5×10^{-4}	.500	.327	2.8	2.4
5	α-Ketoglutarate + malonate	5×10^{-4}	.367	.220	2.4	1.3
6	α-Ketoglutarate + malonate	5×10^{-4}	.367	.307	2.4	2.4
7	α-Ketoglutarate + malonate	1×10^{-3}	.487	.307	2.2	1.3
8	α-Ketoglutarate + malonate	1×10^{-3}	.420	.033	3.0	0.5
9‡	α-Ketoglutarate + malonate	5×10^{-4}	.400	.390	3.1	3.0
10	α-Ketoglutarate + malonate	1×10^{-3}	.300	.187	2.1	0.9
11	α-Ketoglutarate + malonate	1×10^{-3}	.280	.167	3.0	2.7
12	α-Ketoglutarate + malonate	1×10^{-3}	.310	.210	3.9	2.9
13	α-Ketoglutarate + malonate	1×10^{-3}	.268	.193	3.2	2.9
14	β-Hydroxybutyrate	1×10^{-3}	.233	.180	1.5	1.3
15	β-Hydroxybutyrate	1×10^{-3}	.200	.153	2.1	1.9
16	β-Hydroxybutyrate	1×10^{-3}	.173	.153	2.4	2.0
17	β-Hydroxybutyrate	1×10^{-3}	.200	.180	2.4	1.9
18	Succinate	1×10^{-3}	.620	.340	1.9	1.4
19	Succinate	1×10^{-3}	.933	.587	1.9	1.5
20	L-malate	1×10^{-3}	.307	.187	2.6	1.7
21	L-malate	1×10^{-3}	.453	.300	2.2	2.1

* Mitochondria from rat liver.

† Experiments 2 through 8, mitochondria from guinea pig kidney cortex.

‡ Experiments 9 through 21, mitochondria from rabbit kidney cortex.

hexokinase assay. These experiments showed conclusively that phlorizin, in concentrations up to 3×10^{-3} M, had no effect on the reaction velocity of the hexokinase system.

Effect of Electron Carrier Steady State of Mitochondria on Phlorizin Inhibition—In the initial series of experiments (Table I) it was observed that the phlorizin inhibition was quite variable from one experiment to the next, and it soon became apparent that the ability of phlorizin to inhibit respiration was markedly affected by the order in which the reactants were added to the mitochondria.

The effects of ADP, substrate, and oxygen on the oxidation-reduction state of the electron carrier system in mitochondria have been studied by Chance and Williams (13). Freshly prepared mitochondria incubated aerobically without added substrate or ADP were defined by them as being in State I. If ADP was then added, a brief burst of respiration ensued but ended when all endogenous substrate had been utilized. This nonrespiring condition was called State II and was characterized by complete oxidation of the coenzymes in the electron carrier system. State III was obtained by adding substrate to State II mitochondria and was characterized by active respiration and partial reduction of all electron carrier coenzymes. If respiration continued until all ADP was converted to ATP, respiration slowed and State IV was obtained. This was characterized by complete reduction of DPN and complete oxidation of cytochrome *a* with partial reduction of the intermediate coenzymes.

Preliminary experiments had indicated that phlorizin might inhibit more effectively when added to State II than to State IV mitochondria, and it was decided to test this possibility. Warburg flasks with two side arms were used. State II mitochondria were prepared by placing them together with phosphate buffer, $MgSO_4$, cytochrome *c*, ADP, hexokinase, and glucose in the main compartment. Phlorizin was tipped in from the first side arm at 4 minutes and α-ketoglutarate, from the second side arm at 8 minutes. State IV mitochondria were prepared by placing them together with buffer, $MgSO_4$, cytochrome *c*, ADP, and α-ketoglutarate in the main compartment. Phlorizin was again in the first side arm, but the second side arm contained the hexokinase-glucose phosphate acceptor system. Of course, when the second side arm contents were tipped in, the mitochondria were in both cases converted to State III and active respiration occurred.

It may be seen from Table II that 10^{-3} M phlorizin added to mitochondria in State II caused severe inhibition of the respiration initiated by substrate addition, whereas phlorizon added to State IV mitochondria had much less or no effect on respiration occurring upon addition of hexokinase-glucose. It thus appears that mitochondria are much less susceptible to the effects of phlorizin when they are in State IV, *i.e.* when the electron carriers are in the partially reduced state and the adenine nucleotide is in the form of ATP.

Adenine Nucleotide Reversal of Phlorizin Inhibition—Whether the partially reduced state of the electron carriers, the ATP, or both protected the mitochondria could not be directly determined. However, it was decided to study further the effects of ATP for two reasons. (*a*) It had been reported by Lotspeich and Keller (4) that high concentrations of adenine nucleotides could prevent phlorizin inhibition of respiration in guinea pig kidney homogenates. (*b*) Tapley (14) and others have shown that ATP can prevent mitochondrial swelling in hypotonic me-

TABLE II

Effect of electron carrier steady state of mitochondria on phlorizin inhibition

Experiment No.	Oxygen utilization			
	State II*		State IV†	
	Control	Phlorizin 10^{-3} M	Control	Phlorizin 10^{-3} M
	μatoms/min./flask		*μatoms/min./flask*	
1	.76	.37	.77	.75
2	.76	.37	.75	.72
3	.77	.36	.86	.71

* Hexokinase in center; phlorizin added at 4 minutes; α-ketoglutarate at 8 minutes.

† α-Ketoglutarate in center; phlorizin added at 4 minutes; hexokinase at 8 minutes.

dia. This second point will be dealt with in the following paper. The first point, relative to the ability of an excess concentration of adenine nucleotide to reduce the phlorizin inhibition, was verified in mitochondria, as shown in Table III. Control flasks and flasks with 5×10^{-4} M phlorizin were incubated with ATP at 2 mM and 10 mM concentrations. As can be seen, the phlorizin inhibition was reduced from 45 per cent at the low concentration to 23 per cent at the high concentration of ATP.

Thus in mitochondria, as in whole kidney homogenates (4), one can demonstrate the protection against phlorizin inhibition of respiration by excess adenine nucleotide. The experiments presented in the following paper show that ATP prevents the phlorizin-induced swelling of mitochondria in isotonic sucrose and raises the possibility that both the phlorizin inhibition of respiration and the adenine nucleotide reversal can be explained by a membrane permeability effect of phlorizin on mitochondria with resulting changes in the capacity of these structures to maintain normal osmoregulation. Thus the adenine nucleotide reversal of the phlorizin inhibition of respiration would be the result of a prevention of the phlorizin-induced swelling of mitochondria in an "isotonic" environment.

Effect of Malonate on Phlorizin Inhibition—Besides acting as a competitive inhibitor of succinic dehydrogenase, malonate has been shown, like ATP, to prevent the swelling of mitochondria in hypotonic media under certain conditions (14, 15). Since it seems possible, as is shown in the following paper, that the ATP reversal of the phlorizin effect on respiration correlates with the ATP reversal of phlorizin-induced swelling of mitochondria in isotonic sucrose, it was decided to study the effect of phlorizin on α-ketoglutarate oxidation in mitochondria in the absence and presence of malonate. In Table IV are shown the results of such experiments. In these experiments phlorizin was tipped in first from one side arm at 4 minutes; α-ketoglutarate and malonate, from the other side arm at 8 minutes; oxygen uptake measurements were begun at 12 minutes. It is evident from these experiments that the phlorizin inhibition of α-ketoglutarate oxidation in mitochondria is much less when malonate is present than in its absence. This observation may be related to the fact that malonate limits the oxidation of α-ketoglutarate to one step, *i.e.* to succinate. Alternatively, the fact that malonate protects mitochondrial structure and hence the enzymes of oxidative metabolism may bear on this difference.

TABLE III

Reduction of phlorizin inhibition by ATP

Experimental conditions	Oxygen utilization		Inhibition by phlorizin
	Control	Phlorizin 5×10^{-4} M	
	μatoms/min./flask		%
ATP, 2 mM	.58	.32	45
ATP, 10 mM	.53	.41	23

TABLE IV

Effect of malonate on phlorizin inhibition of α-ketoglutarate oxidation by rabbit kidney mitochondria

For experimental details see text.

Experimental conditions	No. of experiments	Oxygen utilization		Per cent of control (mean ± standard error)
		Control	Phlorizin 10^{-3} M	
		μatoms/min./flask		
α-Ketoglutarate	6	.763	.340	44.7 ± 1.7
α-Ketoglutarate + malonate	11	.420	.302	71.6 ± 2.1

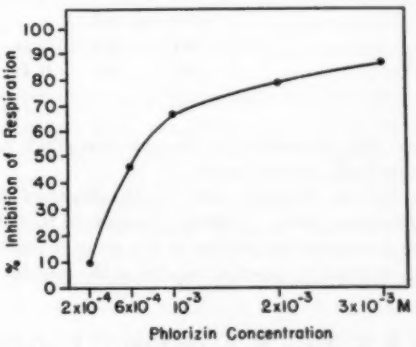

FIG. 1. Relation between phlorizin concentration and inhibition of respiration in kidney cortex mitochondria. α-Ketoglutarate was substrate.

Relation between Phlorizin Concentration and Oxidative Inhibition in Mitochondria—Fig. 1 is a plot of the per cent inhibition of oxygen consumption against phlorizin concentration when phlorizin is added to State II mitochondria. It is evident that the effective range of phlorizin concentrations here is very similar to that seen in the kidney mince (3) and the whole homogenate (4). In the mitochondria, after respiration commenced with the addition of substrate, there was a definite tendency for the respiratory rate to recover from the effects of phlorizin. This effect and its relation to phlorizin concentration are seen in Fig. 2 which is a plot of the per cent inhibition of oxygen utilization against time in minutes after onset of incubation at several different phlorizin concentrations. It appears that at the lowest concentration there is complete recovery of respiration within a few minutes as is seen in the bottom curve; at higher phlorizin concentrations there is only partial recovery or none at all. The capacity to recover from the effects of the inhibitor may be due to the production during respiration of a substance, perhaps ATP, which counteracts the effect of phlorizin. Or perhaps the respiratory enzymes are for

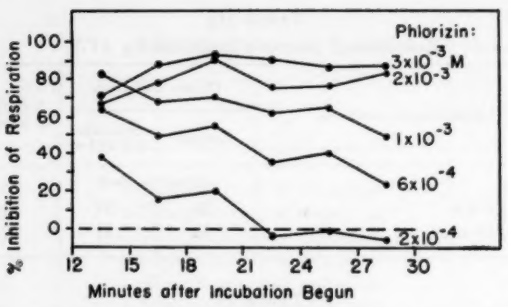

FIG. 2. Experiments showing the varying duration of respiratory inhibition in mitochondria with different phlorizin concentrations. α-Ketoglutarate was substrate.

TABLE V

Effect of phlorizin on oxygen utilization and P:O ratio with and without 2,4-dinitrophenol

Phlorizin added at 4 minutes; α-ketoglutarate and malonate at 8 minutes; oxygen uptake measured between 12 and 22 minutes.

Experimental conditions	Oxygen utilization		P:O	
	Control	Phlorizin 10^{-3} M	Control	Phlorizin 10^{-3} M
	μatoms/min./flask			
No DNP	.484	.376	3.54	2.50
DNP, 10^{-4} M	.420	.301	0.48	0.30

some reason less susceptible to the phlorizin effect when they are in the partially reduced state.

Relation between Phlorizin and 2,4-Dinitrophenol Effects on Oxidative Phosphorylation—Although it appeared that the phlorizin inhibition occurred somewhere in the series of reactions concerned with respiratory electron transfer or the coupled phosphorylations, its effect differed from that of the classical "uncoupler," 2,4-dinitrophenol, in several important respects. Although producing maximal uncoupling, dinitrophenol can stimulate rather than depress respiration; its reduction of the P:O ratio is much more marked than that caused by phlorizin (Table I), and finally the effects of dinitrophenol are not reversed by addition of any of the adenine nucleotides. For these reasons it became of interest to compare further the effects of phlorizin and dinitrophenol on mitochondrial oxidation. The data in Table V are from an experiment designed to compare the effects of phlorizin and dinitrophenol on the one step oxidation of α-ketoglutarate in the presence of malonate. The theoretical P:O ratio for this oxidation is 4. It is seen in Column 4, Table V that the P:O of 3.54 in the control came close to this theoretical figure. Furthermore, dinitrophenol (10^{-4} M) reduced the P:O to 0.48. In the presence of the dinitrophenol, although the P:O was reduced from 2.50 to 0.30, the phlorizin still produced its characteristic inhibition of oxygen utilization (from 4.20 to 3.01). Thus this experiment serves to substantiate what had been surmised, namely, that the phlorizin and dinitrophenol effects on oxidation and phosphorylation are different both in kind and site of action.

SUMMARY

Phlorizin in low concentrations can inhibit oxidation of several substrates and decrease the efficiency of oxidative phosphorylation in kidney and liver mitochondria. The oxidation-reduction state of the electron carrier coenzymes of the mitochondria can markedly alter this phlorizin effect. The adenine nucleotide reversal of the phlorizin inhibition of oxidative metabolism first noted in homogenates has been shown to occur in mitochondria as well. In addition, it has been shown that phlorizin inhibition of α-ketoglutarate oxidation is reduced by malonate. The possibility that this adenosine triphosphate and malonate "protection" is related to the protective effect of these two substances on mitochondrial structure has been discussed.

REFERENCES

1. CRANE, R. K., FIELD, R. A., AND CORI, C. F., *J. Biol. Chem.*, **224**, 649 (1957).
2. CHINARD, F. P., TAYLOR, W. R., NOLAN, M. F., AND ENNS, T., *Science*, **125**, 736 (1957).
3. SHAPIRO, B., *Biochem. J.*, **41**, 151 (1947).
4. LOTSPEICH, W. D., AND KELLER, D. M., *J. Biol. Chem.*, **222**, 843 (1956).
5. KELLER, D. M., AND LOTSPEICH, W. D., *J. Biol. Chem.*, **234**, 991 (1959).
6. COPENHAVER, J. H., JR., AND LARDY, H. A., *J. Biol. Chem.*, **195**, 225 (1952).
7. LOWRY, O. H., AND LOPEZ, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
8. SCHNEIDER, W. C., *J. Biol. Chem.*, **176**, 259 (1948).
9. KUNITZ, M., AND MCDONALD, M. R., *J. Gen. Physiol.*, **29**, 395 (1945–1946).
10. BRAUN, W., WHITTAKER, V. P., AND LOTSPEICH, W. D., *Am. J. Physiol.*, **190**, 563 (1957).
11. NAGAI, K., *Med. J. Osaka Univ.*, **6**, 907 (1956).
12. COLOWICK, S. P., AND KALCKAR, H. M., *J. Biol. Chem.*, **148**, 117 (1943).
13. CHANCE, B., AND WILLIAMS, G. R., *J. Biol. Chem.*, **217**, 409 (1955).
14. TAPLEY, D. F., *J. Biol. Chem.*, **222**, 325 (1956).
15. RAAFLAUB, J., *Helv. Physiol. Acta*, **11**, 142 (1953).

Effect of Phlorizin on the Osmotic Behavior of Mitochondria in Isotonic Sucrose*

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Although phlorizin appears clearly capable of inhibiting respiration and diminishing the efficiency of oxidative phosphorylation (1), the possibility remains that these effects are secondary to a general nonspecific alteration of mitochondrial structure rather than to a single specific inhibition somewhere in the chain of enzymes and coenzymes concerned in oxidative metabolism. For instance, the inhibition of oxidative phosphorylation by the hormone thyroxine, or its metabolites, has been related to some such general alteration of mitochondrial structure (2). Such a structural effect of phlorizin would not only explain its inhibition of mitochondrial metabolism but might harmonize those observations with the known effect that phlorizin has on certain cell membranes.

The studies reported here deal with the osmotic behavior of mitochondria in isotonic sucrose and show that phlorizin promotes the swelling of mitochondria in such an isotonic medium. Furthermore, it shows that ATP and ADP, but not AMP, prevent this phlorizin-induced swelling. These observations are then related to the effects of adenine nucleotides, malonate, 2,4-dinitrophenol, and electron carrier steady state on mitochondrial structure and the phlorizin inhibition of respiration.

EXPERIMENTAL

Since the first observation by Claude (3) that mitochondria swell when placed in hypotonic media, several techniques have been used to study the phenomenon. The changes in mitochondrial structure may be revealed by directly observing the swelling under the phase contrast microscope (4), by a reduction of the dry weight to wet weight ratio (5), or by a decrease in the optical density of the mitochondrial suspensions (6, 7).

In our experiments changes in mitochondrial volume were studied at room temperature by following the optical density changes at 520 mμ in the Beckman model DU spectrophotometer with the use of a technique essentially like that described by Tapley (8). The cuvettes contained 3 ml. consisting of mitochondria, 0.3 M sucrose, and 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.4. Except where indicated, experiments were performed with mitochondria prepared from guinea pig kidney cortex according to the method of Dounce *et al.* (9) which involves 0.001 M ethylenediaminetetraacetate during homogenization and the first washing and a medium of 0.44 M sucrose throughout. Before addition to the spectrophotometer cuvette the final mitochondrial pellet was suspended in sufficient volume of 0.44 M sucrose so that 0.1 ml. of suspension in a final volume of 3 ml. would give an optical density of about 0.5. The mitochondria were used immediately after their preparation.

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RESULTS

Effect of Sucrose Tonicity on Mitochondrial Volume—As may be seen in Fig. 1, guinea pig kidney mitochondria swell very rapidly when placed in hypotonic sucrose so that by the time the first reading is taken at 1 minute swelling is already complete. A rapid swelling in hypotonic media was also found by Cleland (10) for rat heart sarcosomes. However, this may be contrasted to the behavior of rat liver mitochondria which swell slowly over a 10- to 20-minute interval under these conditions (8).

Effect of Phlorizin on Mitochondrial Volume in Isotonic Sucrose—The results from respiration experiments on whole homogenates and respiring mitochondria (1) suggested that phlorizin may cause a general alteration in mitochondrial structure. The curves in Fig. 2 show that although there is no swelling of mitochondria in control vessels, phlorizin causes a definite swelling in the 0.3 M sucrose. Determination of the dry weight to wet weight ratio showed that the decrease in optical density initiated by phlorizin was associated with increase in water content of the mitochondria and thus represented a true swelling from water imbibition. This type of swelling can be distinguished from that induced in hypotonic solutions, because it has a much longer time course. The dose-response relationship for the phlorizin-induced swelling phenomenon is quite comparable to that which exists for the phlorizin-induced block of oxidation; that is, perceptible at 2×10^{-4} M and marked at 10^{-3} M. Furthermore, it was found that phlorizin could initiate additional swelling even in mitochondria which were already severely swollen in a hypotonic medium of 0.06 M sucrose.

Effect of Adenine Nucleotides on Phlorizin-induced Swelling—In view of the finding (1, 11) that sufficient concentrations of ATP can reduce the phlorizin effect on oxidation, it was of particular interest to test the effect of ATP on phlorizin-induced swelling in the mitochondria. In Fig. 3 are shown results of experiments of this type in which it was found that 5×10^{-3} M ATP in the isotonic sucrose medium effectively blocks the swelling induced by 10^{-3} M phlorizin. It has been shown that ATP or the onset of oxidative phosphorylation or both can, under certain conditions, bring about a contraction of swollen mitochondria (12). This might be the basis for the adenine nucleotide reversal of the phlorizin inhibition of respiration in kidney tissue (1). However, as is evident in the experiment of Fig. 4, under the conditions of the present swelling studies, ATP could

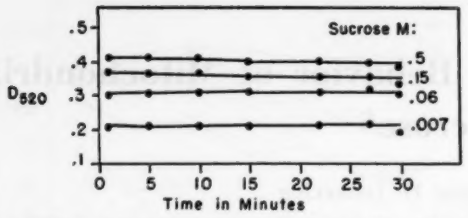

FIG. 1. Effect of sucrose concentration on swelling of guinea pig kidney mitochondria. The only other component of the medium was 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.4. Optical density read against air.

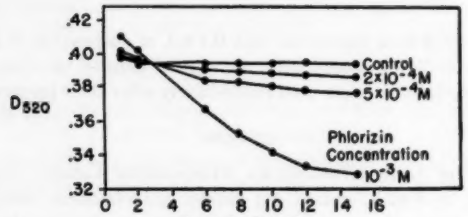

FIG. 2. Swelling of guinea pig kidney mitochondria induced by 2×10^{-4}, 5×10^{-4}, and 10^{-3} M phlorizin in 0.3 M sucrose buffer medium. Optical density read against air.

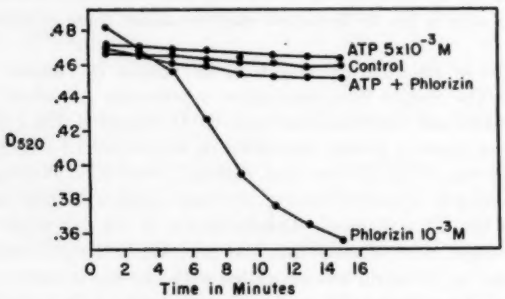

FIG. 3. Effect of ATP on phlorizin-induced mitochondrial swelling in 0.3 M sucrose buffer medium. Optical density read against air.

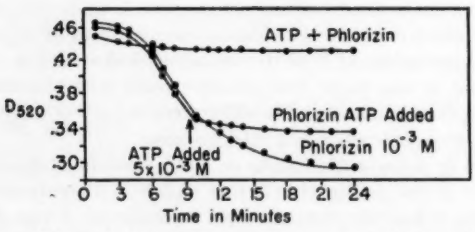

FIG. 4. Effect of added ATP on the course of phlorizin-induced mitochondrial swelling. Sucrose buffer medium, 0.3 M. Optical density was read against a blank and correction made for the dilution caused by adding ATP solution at 9.5 minutes.

only stop the progression of phlorizin-induced swelling and could not completely reverse it and return the mitochondria to their former volume. This observation is in agreement with Tapley's finding (8) that ATP could stop, but not completely reverse, the hypotonicity-induced swelling of rat liver mitochondria.

Comparison of Effects of ATP, ADP, and AMP on Phlorizin

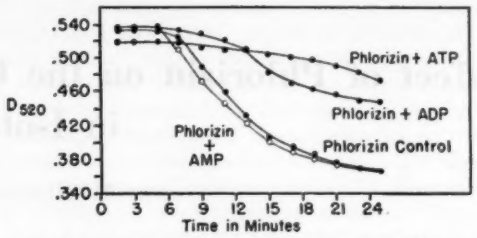

FIG. 5. A comparison of ATP, ADP, and AMP at low concentration, 2×10^{-4} M, on phlorizin-induced mitochondrial swelling. Guinea pig kidney mitochondria suspended in 0.3 M sucrose buffer medium. Optical density read against air.

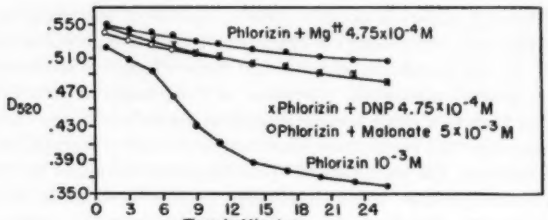

FIG. 6. Effect of malonate, 2,4-dinitrophenol, and magnesium ion on phlorizin-induced mitochondrial swelling. Guinea pig kidney mitochondria suspended in 0.3 M sucrose buffer medium. Optical density read against air.

Swelling in Mitochondria—When it was seen that ADP (5×10^{-3} M) was as effective as ATP in blocking phlorizin-induced swelling (Fig. 4), an attempt was made to reconcile this observation with the finding (1) that mitochondria in a respiratory medium with ADP (State II) are much more susceptible to phlorizin than when they are in a respiratory medium with ATP (State IV). It seemed possible that ADP was acting under the conditions used here by being converted to ATP and AMP by adenylate kinase (13) or in some other way making its high energy phosphate available. In Fig. 5 are plotted data which lend support to this possibility. It may be seen from this experiment that low concentrations of ATP (2×10^{-4} M) forestalled swelling for approximately 15 minutes after the control mitochondria began to swell, whereas ADP in equal concentration (but, of course, containing one half as much high energy phosphate) prevented swelling for only about half as long. And finally, in contrast to the other two adenine nucleotides, AMP, which contains no high energy phosphate, was incapable of preventing the phlorizin-induced swelling.

Effect of Malonate, Dinitrophenol, and Mg^{++}—It has been indicated in the previous paper (1) that 20×10^{-3} M malonate can reduce the effect of phlorizin on oxidative metabolism whereas the phlorizin effect was unaffected by 10^{-4} M dinitrophenol. Both of these agents are known to reduce the swelling of rat liver mitochondria under various conditions (6, 8) so that it became of interest to study their effect, as well as that of phlorizin, on the mitochondrial swelling phenomenon. Fig. 6 is from an experiment which demonstrates that 5×10^{-3} M malonate and 4.75×10^{-4} dinitrophenol, like ATP, are equally effective in counteracting the phlorizin-induced swelling phenomenon. In addition, it may be seen that magnesium ion, an agent which inhibits mitochondrial swelling under other conditions (6), also blocks the phlorizin-induced swelling in isotonic sucrose.

Effect of Phlorizin on Mitochondrial Volume in Respiration Medium—The data presented thus far show good correlation between the dose-response relation for swelling and for inhibition of oxidation, and in addition there is a similarity between the effects of ATP and malonate on both processes. Furthermore, it had been observed in other experiments that phlorizin can promote swelling and that ATP can prevent it even when the 0.3 M sucrose of the medium was replaced by 0.3 M glucose, 0.15 M KCl, or 0.1 M potassium α-ketoglutarate. For these reasons it was decided to study phlorizin-induced swelling in the same medium that had been used in the respiration experiments of the previous study (1) in which a variety of interactions might alter the results considerably.

Fig. 7 is a plot of optical density measurements against time for two such experiments. The experimental conditions for *A* and *B* correspond approximately to the conditions for State II and State IV mitochondrial respiration experiments respectively (1). However, fewer mitochondria were present here to allow for optical density measurements, and the incubation was carried out in the cuvettes of the spectrophotometer at room temperature rather than in the microrespiration flasks. Rabbit kidney mitochondria prepared by the method of Schneider (14) were used. The readings were taken against mitochondrial blanks to allow correction to be made for decrease in optical density due simply to the mitochondrial dilution that occurred with each addition.

It may be seen that upon adding phlorizin in the experiment of *A* (State II) a prompt decrease in optical density occurred. In contrast to this, the addition of phlorizin in the experiment of *B* (State IV) did not change the optical density from the control cuvette. This finding correlates well with the observation (1) that phlorizin inhibits respiration strongly when added to State II mitochondria, but that little or no inhibition occurs when added to State IV. However, the good correlation ends upon conversion to State III by adding substrate in *A* and hexokinase-glucose in *B*. Perhaps the failure of the phlorizinized mitochondria in *B* to behave as the nonphlorizinized, as was the case in respiration experiments, was the result of the unavoidable differences between the experimental conditions used here and those used in the respiration studies.

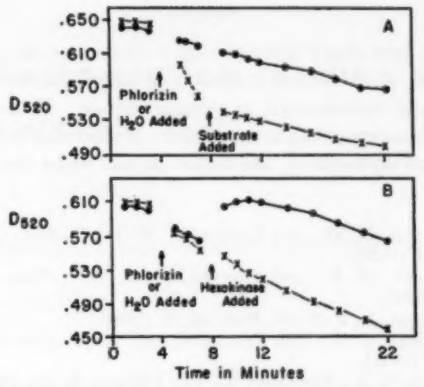

FIG. 7. Effect of phlorizin on optical density of rabbit kidney mitochondria suspended in respiration medium. *A*, phlorizin 10^{-3} M added to mitochondria in presence of ADP and hexokinase (State II). *B*, phlorizin added to mitochondria in presence of ATP and substrate (α-ketoglutarate) (State IV). Readings were taken against a blank and corrections made for dilution of the suspensions. ●, control; ×, phlorizin.

DISCUSSION

Phlorizin in concentrations of 5×10^{-4} to 10^{-3} M can inhibit oxidation and reduce the efficiency of phosphorylation in mitochondria (1). It is believed on the basis of the studies presented above that this inhibition is probably the result of a general structural alteration of mitochondria rather than a specific single enzyme effect. It has been suggested (15) that the maintenance of mitochondrial structure depends upon some active principle in its enzymatic organization. If this is so, phlorizin may act to disrupt and adenine nucleotide to preserve this function. That the metabolism-inhibiting and structure-altering effects are the result of the same action of phlorizin is indicated by the similar dose response range for both effects and the fact that both are diminished by ATP, malonate, and the steady state metabolic conditions for State IV.

Both swelling and decreased efficiency of oxidative phosphorylation occur in the so called aging process (15) of mitochondria, and it seems probable that phlorizin should be grouped along with the several other agents that have been demonstrated to initiate this process. The conclusion that phlorizin acts in this way could be substantiated by testing for the appearance of other phenomena which occur in aging, such as the decreased ability to concentrate certain ions (5) and nucleotides (16), diminished phosphate-oxygen exchange (17), appearance of DPNase (18) and of a magnesium-dependent ATPase (19).

The possible relevance of these findings to phlorizin action *in vivo* is indicated by the report of Nagai (20) who showed that kidney mitochondria from animals chronically treated with phlorizin have a reduced efficiency of oxidative phosphorylation. Von Kossa, quoted by Lusk (21), found severe cloudy swelling of the convoluted tubules of kidneys from rabbits treated with phlorizin. In view of the present interpretation that cloudy swelling is actually due to mitochondrial swelling, this probably indicates that phlorizin, in sufficient concentration, can cause mitochondrial swelling *in vivo*.

Does phlorizin reach concentrations in the proximal tubule cell sufficient to inhibit oxidative metabolism? In a recent study (22) it has been shown that glucose reabsorption is exquisitely sensitive to phlorizin. A total cumulative dose of 2.8 mg. of phlorizin in a 20-kg. dog over a 70-minute period resulted in more than 50 per cent reduction in the tubular capacity to transport glucose. The approximate concentration of phlorizin that has been shown to inhibit oxidative metabolism by 50 per cent *in vitro* is 5×10^{-4} M (1, 11, 23). In order for 2.8 mg. to have this concentration in the renal tubular cells transporting glucose, it would have to occupy a volume of 11.9 ml. Although this volume is probably somewhat larger than the total volume of the cells of the proxmial convoluted tubule, it is obvious that these cells would have to have a very high affinity for phlorizin so that its concentration in other tissues would be many fold lower. Ellinger and Lambrechts (24) have shown that colored glucosuric derivatives of phlorizin are, indeed, concentrated in the cells of the proximal tubule. But it must be remembered that some of the phlorizin administered to the dog was no doubt lost through excretion by the kidneys (25) and liver (26). This argument indicates that a phlorizin concentration of 5×10^{-4} M in the cells of the proximal tubule is in the realm of possibility but that a direct measure of phlorizin concentration here is certainly needed.

SUMMARY

It has been shown that phlorizin in concentrations comparable to those which inhibit oxidation in mitochondria also cause swelling of mitochondria in isotonic sucrose. Phlorizin is less able to induce swelling in the presence of adenosine triphosphate, adenosine diphosphate, and malonate, and under the metabolic conditions that obtain when phlorizin is added to mitochondria in the presence of ATP and α-ketoglutarate. These effects on phlorizin-induced swelling correlate well with the effects of these agents and conditions on the phlorizin inhibition of oxidation. It thus appears that the phlorizin effect on mitochondrial metabolism may be secondary to a general effect on mitochondrial structure exerted most probably at the membrane.

REFERENCES

1. Keller, D. M., and Lotspeich, W. D., *J. Biol. Chem.*, **234**, 987 (1959).
2. Tapley, D. F., and Cooper, C., *J. Biol. Chem.*, **222**, 341 (1956).
3. Claude, A., *J. Exptl. Med.*, **84**, 51 (1946).
4. Harman, J. W., and Feigelson, M., *Exptl. Cell Research*, **3**, 47 (1952).
5. Price, C. A., Fonnesu, A., and Davies, R. E., *Biochem. J.*, **64**, 754 (1956).
6. Raaflaub, J., *Helv. Physiol. Acta*, **11**, 142 (1953).
7. Malamed, S., and Recknagel, R. O., *Proc. Soc. Exptl. Biol. Med.*, **98**, 139 (1958).
8. Tapley, D. F., *J. Biol. Chem.*, **222**, 325 (1956).
9. Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S., and Cottone, M. A., *J. Biophys. Biochem. Cytol.*, **1**, 140 (1955).
10. Cleland, K. W., *Nature*, **170**, 497 (1952).
11. Lotspeich, W. D., and Keller, D. M., *J. Biol. Chem.*, **222**, 843 (1956).
12. Chappell, J. B., and Perry, S. V., *Nature*, **173**, 1094 (1954).
13. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191**, 485 (1951).
14. Schneider, W. C., *J. Biol. Chem.*, **176**, 259 (1948).
15. Ernster, L., and Lindberg, O., *Annual Review of Physiology, Vol. 20*, Annual Reviews, Inc., Palo Alto, California, 1958, p. 13.
16. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **215**, 221 (1955).
17. Boyer, P. D., Luchsinger, W. W., and Falcone, A. B., *J. Biol. Chem.*, **223**, 405 (1956).
18. Hunter, F. E., Jr., and Ford, L., *J. Biol. Chem.*, **216**, 357 (1955).
19. Potter, V. R., Siekevitz, P., and Simonson, H. C., *J. Biol. Chem.*, **205**, 893 (1953).
20. Nagai, K., *Med. J. Osaka Univ.*, **6**, 907 (1956).
21. Lusk, G., *Ergeb. Physiol.*, **12**, 315 (1912).
22. Lotspeich, W. D., and Woronkow, S., *Am. J. Physiol.*, **195**, 331 (1958).
23. Shapiro, B., *Biochem. J.*, **41**, 151 (1947).
24. Ellinger, P., and Lambrechts, A., *Compt. rend. soc. biol.*, **124**, 261 (1937).
25. Braun, W., Whittaker, V. P., and Lotspeich, W. D., *Am. J. Physiol.*, **190**, 563 (1957).
26. Jenner, F. A., and Smyth, D. H., *J. Physiol.*, **137**, 18P (1957).

Phlorizin Inhibition of the Insulin Expansion of the Galactose Space in the Eviscerate Rat*

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Several reports have recently appeared which indicate that phlorizin may act at certain cell membranes to inhibit penetration of them by glucose. A recent study by Krane and Crane (1) showed that in slices of rabbit kidney cortex, phlorizin can inhibit the rate of active accumulation of galactose from the medium. Under anaerobic conditions or in the presence of an uncoupler of oxidative phosphorylation, galactose penetration into the slice occurred by simple diffusion, but, nevertheless, phlorizin inhibited the rate at which this equilibrium was obtained. Thus these workers believed that phlorizin was not affecting any of the reactions furnishing energy for an active sugar transport. Chinard *et al.* (2), using a rapid injection into the renal artery in the dog, presented evidence that phlorizin causes the luminal side of the tubular cells to become impermeable to glucose.

Two additional reports dealing with other tissues also indicate a cell membrane site of action for phlorizin. Crane *et al.* (3) showed that the rate of penetration of 3-methyl glucose into Ehrlich ascites tumor cells was decreased by phlorizin, although "no" phlorizin entered the cells. Wilbrandt (4), on the basis of extensive kinetic studies, put forth the hypothesis that phlorizin inhibits glucose transport across the red cell membrane by blocking an enzyme which cleaves glucose from a membrane carrier.

In view of these considerations indicating that phlorizin may act directly to block the mechanism by which monosaccharides penetrate cell membranes, rather than directly to block the source of energy for transport, it was believed that it would be of interest to determine whether phlorizin has any more general effect in the body on membrane permeability to sugars. We wondered, for instance, whether it might act in such a general way as to alter the permeability of muscle cells to sugars. To test this question, it was decided to utilize the observation of Levine *et al.* (5) who observed that in the eviscerated, nephrectomized dog insulin could expand the galactose volume of distribution (galactose space) from one approximating the extracellular fluid volume to one equalling the volume of total body water. It was found that although phlorizin itself had no effect on the galactose space in the eviscerate rat, under certain conditions phlorizin could inhibit strikingly the insulin effect of expanding the galactose space. The purpose of the present paper is to present the evidence supporting this conclusion and to discuss it in the light of a membrane site for phlorizin action.

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† This work was performed during the tenure of a Life Insurance Medical Research Fund Postdoctoral Fellowship.

EXPERIMENTAL

Measurements of galactose space were carried out in 300-gm. eviscerated, nephrectomized white rats. Functional evisceration was done by the method of Russell (6) under Nembutal anesthesia. Since it has been shown that galactose is not measurably metabolized in the eviscerate preparation (5), its volume of distribution in such an animal can be used to study changes in cell permeability to galactose. Blood glucose levels were maintained in all eviscerate animals by giving a solution of 25 mg. of glucose per 100 gm. of body weight subcutaneously each hour. The design of each experiment is included in the legends of Tables I and II below. The dose of phlorizin employed was selected because it approximates that which has been shown to cause complete blockage of glucose reabsorption in the kidney (7). All blood samples were drawn from the inferior vena cava into heparinized syringes and centrifuged. Plasma galactose was determined by the photometric method of Nelson (8) with yeasting of the filtrates and galactose standards (9). Plasma concentrations were divided by 0.94 to convert to plasma water concentrations. The volume of the galactose space was taken as the ratio of the amount of galactose administered to the concentration of galactose in the plasma water, and this is expressed as a per cent of total body weight.

RESULTS

Effect of Insulin on Galactose Space—Table I shows data from an experiment which confirms the finding of Levine *et al.* (5) that insulin does, indeed, enlarge the galactose space. It is evident that this effect is apparent at 1 hour but is most marked at 2 hours. Thus at this time the control group of animals had a galactose space which was 28 ml./100 gm. of body weight, the approximate volume of the chloride space. In the insulin-treated animals, on the other hand, the galactose space was 45 ml./100 gm. of body weight, an increase of about 60 per cent above control levels. The magnitude of this increase in galactose space after insulin corresponds well with that found by Levine *et al.* in dogs. Since the eviscerate animal is essentially a muscle preparation, this effect has been interpreted as being due to an insulin action on the muscle cell membrane to facilitate galactose penetration (10).

Effect of Phlorizin on Insulin Expansion of Galactose Space—A preliminary series of experiments showed quite conclusively that phlorizin itself, in doses sufficient to produce maximal glycosuria in the rat, had no effect at all on the galactose space in the eviscerate preparation. Therefore, it was decided to carry out a second series of experiments to determine whether phlorizin affected the ability of insulin to expand the galactose space.

TABLE I

Expansion of galactose space by insulin in eviscerate rat

Galactose, 70 mg./100 gm. in volume of 0.5 ml. of 0.15 M $NaHCO_3$ per 100 gm. injected in inferior vena cava at zero time. Insulin (Squibb, regular), 3.2 units, injected subcutaneously at zero time. Two-ml. blood samples drawn at 1 and 2 hours for galactose determination.

Animal No.	Galactose space			
	Controls		Insulin	
	1 hr.	2 hrs.	1 hr.	2 hrs.
	ml./100 gm. body weight		*ml./100 gm. body weight*	
1	25.5	27.2	38.3	43.1
2	25.2	26.6	42.4	46.9
3	25.7	31.0	37.6	44.8
Mean.....	25.5	28.3	39.4	44.9

TABLE II

Inhibition by phlorizin of insulin-induced expansion of galactose space in eviscerate rat

Galactose, 70 mg./100 gm. in 0.15 M $NaHCO_3$, injected into inferior vena cava at zero time. In test animals this solution also contained phlorizin, 14 mg./100 gm. Volume of injected solution was 0.5 ml./100 gm. of body weight. At 30 minutes 0.4 unit of regular insulin (Squibb) was given intravenously and 2.8 units subcutaneously. Blood samples were drawn at 2 hours for galactose determination.

$t = 5.82$; level of significance of difference between means, $p < 0.001$.

Animal No.	Galactose space	
	Insulin	Insulin + phlorizin
	ml./100 gm. body weight	
1	51.7	35.4
2	48.4	30.6
3	59.4	46.1
4	59.3	37.4
5	45.1	45.1
6	58.4	41.8
7	55.7	31.3
8	46.9	30.2
9	51.1	33.7
Mean.....	52.9	36.8
S.D.....	5.52	6.20

In Table II are shown the results of these studies with nine animals in each group. The data show that the animals which received phlorizin and insulin had a significant ($p < 0.001$) lower volume of distribution of galactose than the animals which received insulin alone. Thus the galactose space in the insulin-phlorizin group was 36.8 ml./100 gm. of body weight, a figure not much above the galactose space in the noninsulinized, non-phlorizinized animal (Table I). However, the control group of animals, treated in an identical manner but not given phlorizin, had a mean galactose space of 52.9 ml./100 gm. of body weight. Thus it appears that phlorizin, in doses used here, is capable of blocking almost completely the now familiar insulin-induced expansion of the galactose space in the eviscerate rat.

It may be calculated that the dose of phlorizin given (14 mg./100 gm. of eviscerated body weight) would result in a maximal final phlorizin concentration of about 2×10^{-3} M if it were entirely limited to the extracellular fluid volume (as measured by the inulin space); or if it were distributed throughout the entire body water, it would result in a minimal concentration of about 4×10^{-4} M. Thus the concentration which blocks the insulin effect on galactose distribution is approximately in the range found to promote mitochondrial swelling in isotonic sucrose (11) as noted in the previous paper.

DISCUSSION

The data presented here show that whereas phlorizin alone is not able to affect the penetration of galactose into cells, it is able to prevent in a rather striking way the facilitation of galactose penetration caused by insulin. The exact meaning of this observation is not obvious from the present data and will depend, no doubt, on a better understanding of the action of insulin. A speculative discussion of how insulin might alter the cell permeability to sugars has recently been presented by Krahl (12). Insulin is a polypeptide of known structure which may combine with the lipoproteins of the cell membrane and either alter the "pore" size and thus the diffusion permeability or alter the activity of a membrane "carrier" and thus facilitate active transport across the cell membrane.

That phlorizin itself is capable of combining with proteins on the surface of certain cells has recently been shown. Rodriguez and Osler (13) have found that the lysis of sensitized sheep erythrocytes by guinea pig complement is markedly inhibited by phlorizin in concentrations of 10^{-2} M to 10^{-4} M. The mechanism of this inhibition indicates a cell surface competition between phlorizin and one of the complements for the sites on the sensitized erythrocytes.

Whatever the ultimate explanation of the phlorizin effect in blocking an insulin expansion of galactose space, the observation adds another link to the chain of evidence supporting a membrane site for the action of this glycoside and provides another experimental tool for the investigation of the nature of its interaction with cell membranes. That its gross biological effects in the intact animal seem to be limited mainly to sugar transport in kidney and intestine remains an unsolved problem.

SUMMARY

Experiments with the functionally eviscerate rat have shown that phlorizin, although without effect on the galactose space when given alone, is capable of preventing the usually observed expansion of this galactose space produced by insulin. This observation is presented and discussed in terms of a membrane-carrier site for the action of phlorizin in inhibiting the biological transport of sugars.

REFERENCES

1. KRANE, S. M., AND CRANE, R. K., *Federation Proc.*, **16**, 206 (1957).
2. CHINARD, F. P., TAYLOR, W. R., NOLAN, M. F., AND ENNS, T., *Science*, **125**, 736 (1957).
3. CRANE, R. K., FIELD, R. A., AND CORI, C. F., *J. Biol. Chem.*, **224**, 649 (1957).
4. WILBRANDT, W., *Active transport and secretion*, Cambridge University Press, New York, 1954, pp. 136–162.

5. Levine, R., Goldstein, M. S., Huddlestun, B., and Klein, S. P., *Am. J. Physiol.*, **163**, 70 (1950).
6. Russell, J. A., *Am. J. Physiol.*, **136**, 95 (1942).
7. Smith, H. W., *The kidney: structure and function in health and disease*, Oxford University Press, New York, 1951, p. 98.
8. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
9. Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, **83**, 51 (1929).
10. Levine, R., and Goldstein, M. S., *Recent Progr. in Hormone Research*, **11**, 343 (1955).
11. Keller, D. M., and Lotspeich, W. D., *J. Biol. Chem.*, **234**, 991 (1959).
12. Krahl, M. E., *Perspectives in Biol. and Med.*, **1**, 69 (1957).
13. Rodriguez, E., and Osler, A. G., *Federation Proc.*, **17**, 533 (1958).

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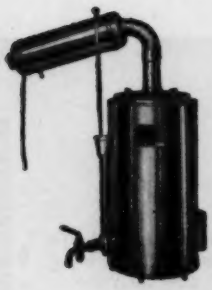

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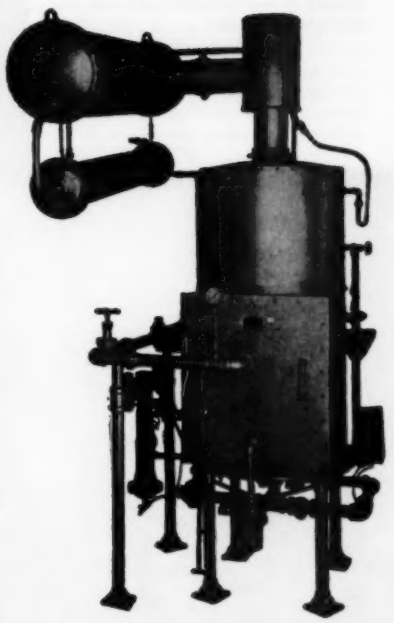

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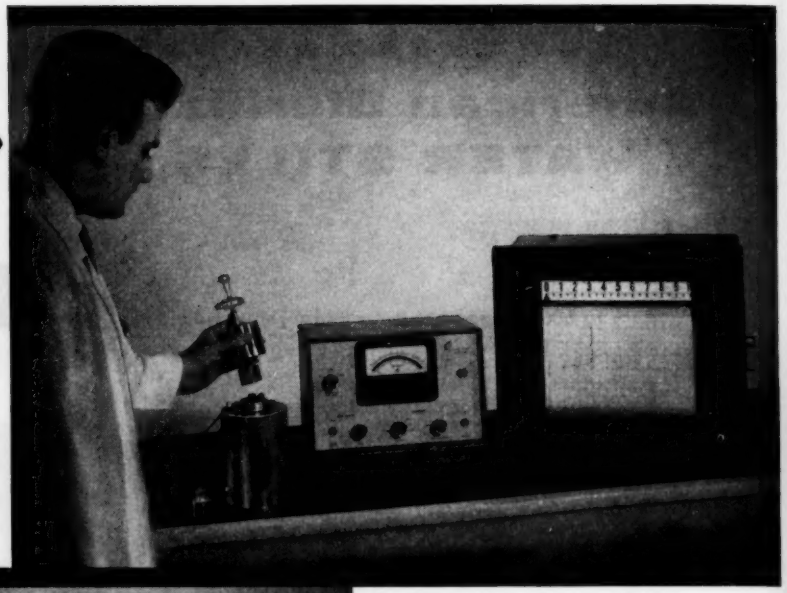

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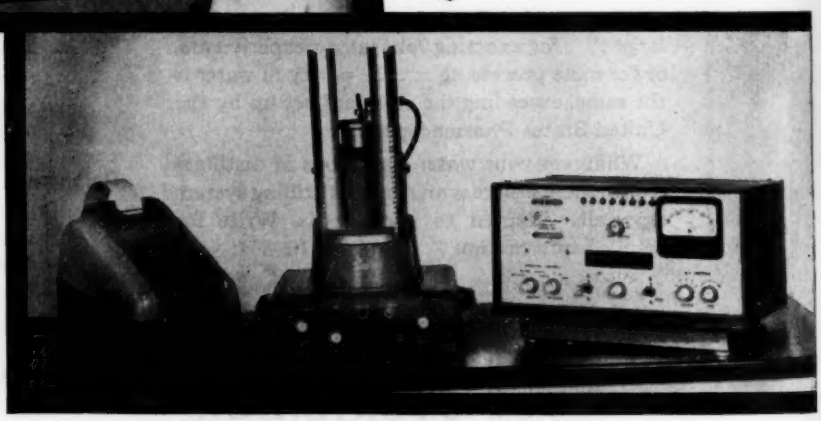

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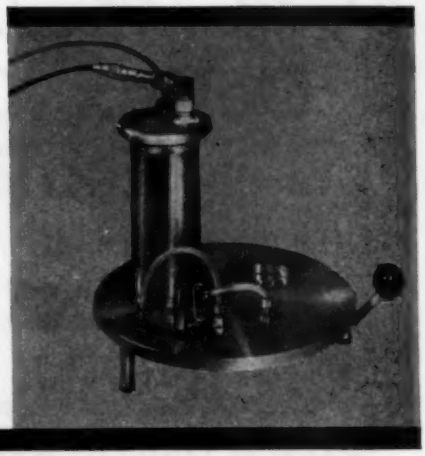

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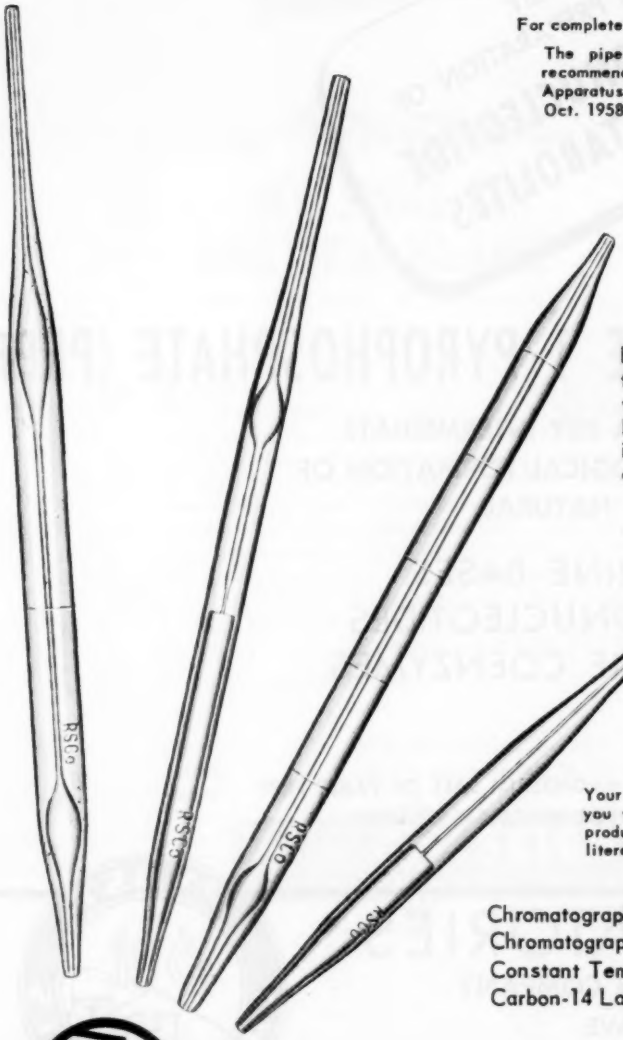

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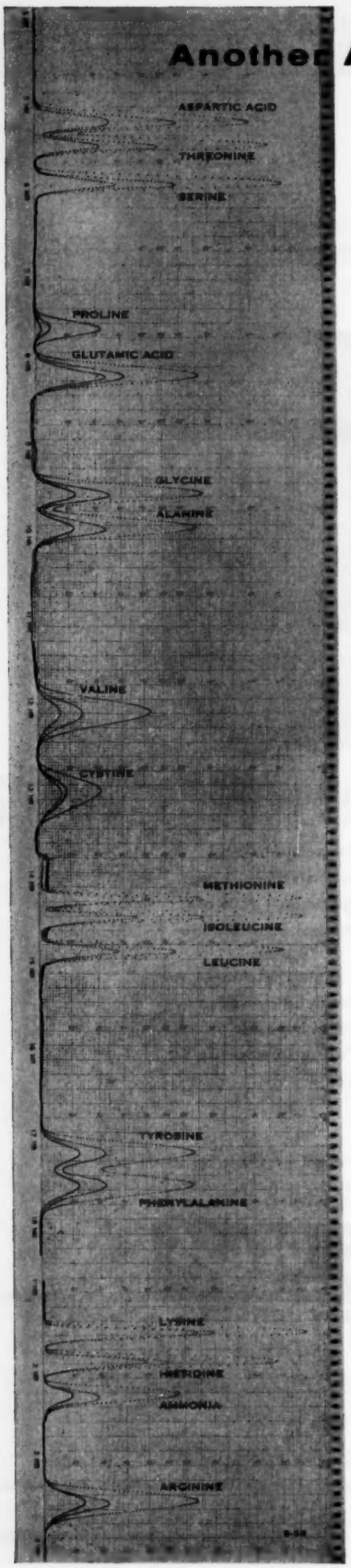

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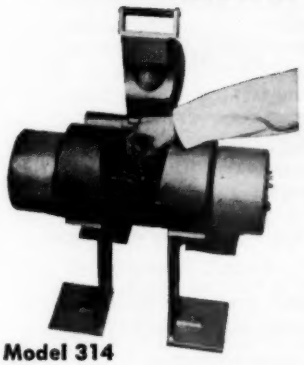

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